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(54) Title: ISOLATED FrpB NUCLEIC ACID MOLECULE AND VACCINE

(57) Abstract

The present invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein. The invention also provides vaccine compositions capable of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis* comprising the FrpB protein encoded by the isolated nucleic acid of the invention and a pharmaceutically acceptable carrier.

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ISOLATED FrpB NUCLEIC ACID MOLECULE AND VACCINE

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BACKGROUND OF THE INVENTION

FrpB has been described as a 70 kD major iron-regulated, outer-membrane protein common to *N. gonorrhoeae* and *N. menigitidis* (16, 21). The iron uptake systems of *N. meningitidis* and *N. gonorrhoeae* are similar (3,17).

Previous studies showed that FrpB is surface exposed and immunogenic *in vivo* (1,16, 41). Polyclonal and some monoclonal anti-FrpB antibodies recognize the denatured protein on Western blots of nearly all gonococcal and meningococcal isolates tested (16 and this invention). Other monoclonal antibodies directed against meningococcal FrpB are bactericidal and strain specific (41). Nevertheless, the size of FrpB appears to be well conserved.

FrpB is useful as a vaccine because of its surface exposure (1,16,41), partial antigenic conservation (8,16), and susceptibility to attack by bactericidal antibodies (41). The cloning and sequencing of the *frpB* gene of this invention has made possible the

production of a vaccine against infection in mammals by *N. gonorrhoeae* or *N. meningitidis*.

SUMMARY OF THE INVENTION

The present invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein.

The invention also provides a method of producing a vaccine composition that protects a mammal from infection by *N. gonorrhoeae* or *N. meningitidis* comprising combining the FrpB protein encoded by the isolated nucleic acid of the invention with a pharmaceutically acceptable carrier.

The invention further provides a vaccine composition capable of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of the invention and a pharmaceutically acceptable carrier.

In addition, the invention provides antibodies directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of the invention.

The invention also provides a method of detecting an antibody specific for *N. gonorrhoeae* or *N. meningitidis* in a sample comprising contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of the invention under conditions to form a complex between the polypeptide and the antibody; and detecting any complex so formed.

Furthermore, the invention provides a method of treating a mammal infected by *N.*

gonorrhoeae or *N. meningitidis* comprising administering to the mammal an antibody of the invention, wherein the antibody is directed to an epitope of an *N. gonorrhoeae* or *N. meningitidis* FrpB protein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 Oligonucleotide MB.3 is shown 3' to 5' and corresponds to non-coding strand. The *frpB* sequence presented in this figure is deposited with GenBank under the accession number U13980.

FIG. 2 Restriction map of *frpB* clones. The position of the *frpB* ORF is indicated below the physical map by the stippled box. Only relevant cloning sites are shown C, *Cla* I; D, *Dra* I; E, *EcoR* I; M, *Mlu* I. Also shown is the position of oligonucleotide MB.3, which was deduced from the amino-terminal amino acid sequence of the mature protein.

FIG. 3 Nucleotide sequence of the gonococcal *frpB* gene from strain FA19. Single letter codes for deduced amino acid sequence are shown below the nucleotide sequence. Asterisk indicates termination codon. Solid bar below nucleotide sequence indicates putative Fur box. Putative -10 and -35 sequences are boxed. RBS indicates ribosome binding site. Solid triangle shows *Bgl* I site of Ω insertion. Vertical arrow indicates signal peptidase I cleavage site. Inverted horizontal arrows indicate inverted repeat.

FIG. 4 Southern-blot analysis of FA19 and FA6807 DNA. Panel A was probed with pUNCH319-specific fragment. Panel B was probed with the Ω fragment. Lanes 1 contain FA19 DNA digested with HincII and lanes 2 contain FA6807 DNA digested with HincII. Ω fragment is 2kb. Molecular weight markers are shown in kilobases (kB).

FIG. 5 Western blot of FA19 and FA6807 membranes. Blot was probed with anti-FrpB monoclonal antibody, W.6. Lanes 1 and 2 are FA19; lanes 3 and 4 are FA6807. Lanes 1 and 3 contain total membranes prepared from iron-sufficient cultures; lanes 2 and 4 contain total membranes from iron-deficient cultures. Approximate locations of molecular mass standards are indicated at left in kilodaltons.

FIG 6 Growth of FA19 and FA6807 in CDM in the presence of variable concentrations of aerobactin. Graph A represents FA19; graph B represents FA6807. (filled-in Δ), 100uM citrate; (■), 2.5uM Tf; (Δ), 3uM aerobactin; (●). 1uM aerobactin; (□), 0.3uM aerobactin; and (O), no iron source.

FIG. 7 ^{55}Fe uptake from ^{55}Fe-heme and ^{55}Fe-Tf. Solid columns represent mean uptake from heme and open columns represent mean uptake from Tf. 100% uptake determined from average FA19 uptake experiment. Standard deviations are indicated by error bars. Genotypes are FA19 wild type, FA6807 (frpB), and FA6747 (tpbA).

FIG 8 Reconstruction of *frpB* in pACYC184. Relevant sites are B, *Bam*H I; C, *Cla* I; D. *Dra* I; M, *Mlu* I; and X, *Xba* I. Solid arrow represents chloramphenicol acetyl transferase (Cm), stripped arrow represents tetracycline resistance gene (Tc), solid bar represents pACYC184 origin of replication (Ori), stippled boxes represent *frpB* coding sequences, stippled arrow indicates entire *frpB* coding regions, open boxes represent DNA 5' and 3' of *frpB*. *frpB'* and *frpB''* represent partial *frpB* coding sequences.

FIG. 9 Growth of RK1065 (pACYC184) and RK1065 (pUNCH331) on heme plates. Plate 1 contains heme only. Plate 2 contains heme and d-aminolevulinic acid. A is RK1065 (pACYC184) and B is RK1065 (pUNCH331). Antibiotic discs are E., erythromyocin; N, novobiocin; and R, rifampicin.

FIG. 10 Nucleotide sequence of the gonococcal *frpB* gene from strain FA1090. The three letter codes for deduced amino acid sequence are shown below the nucleotide sequence. Three asterisks indicate termination codon.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising at least a portion of a FrpB protein. In one embodiment of this invention, the isolated nucleic acid molecule is DNA. In other embodiments of this invention, the isolated nucleic acid molecule is cDNA or RNA. In a preferred embodiment of this invention, the isolated nucleic acid molecule comprises a sequence that is the same as or substantially the same as at least a portion of the nucleotide sequence shown in Figure 3. In a more preferred embodiment, the isolated nucleic acid molecule comprises a sequence that is the same as the nucleotide sequence shown in Figure 3.

The invention also provides a FrpB protein comprising the amino acid sequence encoded by the isolated nucleic acid molecules described above. Preferably, the amino acid sequence encodes an antigenic, and more preferably, an immunogenic FrpB. As used herein, antigenic means that the FrpB induces specific antibodies in a mammal, and immunogenic means that the FrpB induces an immune response in a mammal.

As used herein, the term "FrpB" means Fe-regulated protein B and encompasses any polypeptide having an amino acid sequence identical, or substantially identical, to the amino acid sequence of a naturally-occurring FrpB, as well as antigenic fragments thereof. The FrpB nucleic acid and amino acid sequences in the various strains of *N. gonorrhoeae* and *N. meningitidis* are homologous, but exhibit slight differences in their sequences, for example, the nucleic acid and amino acid differences between the homologous strains FA19 and FA1090 shown in Figure 3 and Figure 10, respectively.

In addition, FrpB encompasses equivalent antigenic polypeptides whose amino acid sequence varies from a naturally-occurring FrpB by one or more amino acid, either

internally such as a point mutation, or by addition or deletion at the COOH terminus or NH_2 terminus or both. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by one or more substitutions, additions and/or deletions, is considered to be an equivalent sequence. Preferably, less than 25%, more preferably less than 10%, and most preferably less than 5% of the number of amino acid residues in a sequence are substituted for, added to, or deleted from the proteins of the invention.

For example, it is known to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids generally considered to be equivalent are:

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);
(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V); and
(e) Phe(F) Tyr(Y) Trp(W).

Such FrpB equivalents include analogs that induce an immune response in a mammal comparable to that of natural FrpB. In addition, such equivalents are immunologically cross-reactive with their corresponding FrpB protein.

A FrpB protein fragment preferably contains sufficient amino acid residues to define an epitope of the antigen. The fragment may, for example, be a minigene encoding only the epitope. Methods for isolating and identifying immunogenic fragments from known immunogenic proteins are described by Salfeld et al. (72) and by Isola et al. (73).

If the fragment defines a suitable epitope, but is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet

hemocyanin, Ig sequences, TrpE. and human or bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

In a preferred embodiment, FrpB of FA19 is or is an equivalent of the approximately 73 kD outer membrane FrpB protein that is part of the iron regulon of *Neisseria gonorrhoeae* or of *Neisseria meningitidis*. Determinations whether two amino acid sequences are substantially homologous may be based on FASTA searches in accordance with Pearson and Lipman (74).

The FrpB of the present invention may be prepared by methods known in the art. Such methods include, for example, (a) isolating FrpB directly from *Neisseria gonorrhoeae* or *Neisseria meningitidis;* and (b) using the nucleic acid molecule of the invention encoding FrpB to produce recombinant FrpB.

(a) Direct Isolation of FrpB:
The FrpB may be isolated directly from *Neisseria gonorrhoeae* or *Neisseria meningitidis* by methods known in the art. First, gonococcal or meningococcal outer membranes are isolated and prepared by known methods. The methods described by West and Sparling (75) and by Schryvers and Morris (76) are suitable.

The isolated membrane FrpB proteins or fragments may be solubilized by known methods, such as the addition of detergents. Commonly used detergents include Octyl-B-Glucoside, Chaps, Zwittergent 3.14 or Triton-X. The use of detergents to enhance solubility of membrane proteins is described by Jones et al. (77), Helenius et al. (78), and Hjelmeland and Chrambach (79).

The FrpB proteins or fragments are isolated from the solubilized membrane fraction by standard methods. Some suitable methods include precipitation and liquid

chromatographic protocols such as ion exchange, hydrophobic interaction and gel filtration. See, for example, Methods Enzymol. (80) and Scopes (81).

Purified material may also be obtained by separating the protein or fragment on preparative SDS-PAGE gels, slicing out the band of interest and electroeluting the protein from the polyacrylamide matrix by methods known in the art. The detergent SDS is removed from the protein by known methods, such as by dialysis or the use of a suitable column, such as the Extracti-Gel column from Pierce.

(b) Using Nucleic Acid Molecule of the Invention to Produce FrpB:
Alternatively, recombinant methods known in the art may be used for preparing FrpB. For example, FrpB may be produced from the isolated or synthesized nucleic acid molecule of the invention that encodes at least a portion of FrpB; cloning the DNA in a suitable host; expressing the DNA in the host; and harvesting FrpB. (See Sambrook et al. (82)).

Using standard methods of nucleic acid isolation, DNA can be obtained from strains that have been deposited with the American Type Culture Collection, Rockville, Maryland. FA1090 (ATCC Accession No.) was deposited on April 8, 1996, in accordance with the Budapest Treaty. Strain FA19 (ATCC Accession No. 55073) was deposited earlier on July 12, 1996, also in accordance with the Budapest Treaty.

The DNA may also be synthesized chemically from the four nucleotides in whole or in part by methods known in the art. Such methods include those described by Caruthers in Science 230, 281-285 (1985).

If necessary a full length DNA may also be produced by preparing overlapping double-stranded oligonucleotides, filling in the gaps, and ligating the ends together. The DNA may be cloned in a suitable host cell and expressed. The DNA and protein may be recovered

from the host cell. See, generally, Sambrook et al, "Molecular Cloning," Second Edition. Cold Spring Harbor Laboratory Press (1987).

The invention provides a vector which comprises the nucleic acid molecule described above which encodes an amino acid sequence comprising at least a portion of FrpB. Suitable vectors comprise, but are not limited to, a plasmid or a virus. This vector may be transfected into a suitable host cell to form a host vector system for the production of FrpB or of a polypeptide having the biological activity of at least a portion of a FrpB antigenic polypeptide.

Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic vectors include plasmids from E. coli, such as colE1, pCR1, pBR322, pMB9, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13, f1, and other filamentous single-stranded DNA phages.

Vectors for expressing proteins in bacteria, especially E.coli, are also known. Such vectors include pK233 (or any of the tac family of plasmids), T7, and lambda P_L. Examples of vectors that express fusion proteins include the PATH vectors described by Dieckmann and Tzagoloff (83). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); and glutathione S-transferase (pGST) - see Gene (84) and Peptide Research (85).

Vectors useful in yeast are available. A suitable example is the 2μ plasmid.

Suitable vectors for use in mammalian cells are also known. Such vectors include well-

known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences and vectors derived from combination of plasmids and phage DNA.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg (86); S. Subramani et al (87); R.J. Kaufmann and P.A. Sharp (88); S.I. Scahill et al (89); G. Urlaub and L.A. Chasin (90).

The expression vectors preferably contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of f1 coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Suitable expression hosts include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRCI, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

VACCINES

FrpB encoded by a nucleic acid molecule of this invention has particular utility as a vaccine that protects a mammal from infection by *N. gonorrhoeae* or *N. meningitidis*, since the FrpB unexpectedly induces an effective immune response when presented to the immune system that protects from or prevents infection by *N. gonorrhoeae* or *N. meningitidis*. To protect from infection by *N. gonorrhoeae*, the FrpB is preferably substantially the same, as defined above, as at least a portion of the FrpB of *N. gonorrhoeae*. To protect from infection by *N. meningitidis*, the FrpB is preferably substantially the same, as defined above, as at least a portion of the FrpB of *N. meningitidis*. The immune response may also produce a therapeutic effect in an already infected mammal. The mammal is preferably a human.

The invention provides a vaccine composition which comprises the FrpB protein encoded by a nucleic acid of the invention and a pharmaceutically acceptable carrier, such as saline, sterile water, phosphate buffered saline solution, liposomes and emulsions. Other buffering and dispersing agents and inert non-toxic substances suitable for delivery to a mammal may be incorporated in the vaccine composition and are well known to those skilled in the art. The compositions may be sterilized by conventional sterilization techniques.

Adjuvants, which facilitate stimulation of the host's immune response, may be used in the vaccine compositions. Such adjuvants may include, for example, muramyl peptides, lymphokines, such as interferon, interleukin-1 and interleukin-6, or bacterial adjuvants. The adjuvant may comprise suitable particles onto which the mutant or wild-type FrpB protein is adsorbed, such as aluminum oxide particles. These vaccine compositions containing adjuvants may be prepared as is known in the art.

The concentration of FrpB in the composition may vary depending on, for example, fluid volume or antigenicity, and in accordance with the particular mode of administraton chosen.

The invention further provides a method of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis* comprising administering to the mammal the vaccine composition of the invention. The vaccine may be administered to a mammal by methods known in the art. Such methods include, for example, oral, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, or intradermal administration.

This invention also provides a method of producing the above vaccine composition by combining FrpB with a pharmaceutically acceptable carrier, and preferably, also with an adjuvant, as defined above.

FrpB ANTIBODIES

The invention provides antibodies raised against FrpB epitopes encoded by at least a portion of the isolated nucleic acid sequence of the invention. The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein (91) and the recombinant DNA method described by Huse et al. (92).

Mammals infected with *N. gonorrhoeae or N. meningitidis* may be treated by administering an antibody of the invention. Preferably, an antibody raised against a polypeptide comprising an amino acid sequence present in *N. gonorrhoeae or N. meningitidis* is preferred.

For therapeutic purposes, the antibodies are preferably neutralizing antibodies that

significantly inhibit the growth of or kill the bacterial cells *in vitro* or *in vivo*. Growth of the bacteria is significantly inhibited *in vivo* if the inhibition or neutralization is sufficient to prevent or reduce the symptoms of the disease of a mammal infected with the disease.

Neutralizing antibodies may also be used to produce anti-idiotypic antibodies useful as vaccines for immunizing mammals infected with *N. gonorrhoeae* or *N. meningitidis*. Anti-idiotypic antibodies are prepared in accordance with methods known in the art.

DETECTING FrpB USING PROBES

The invention also provides a method of detecting FrpB in a sample using a probe specific for a FrpB polypeptide. The probe may be an antibody described above. Methods are known for detecting polypeptides with antibodies. For example, a polypeptide may be immobilized on a solid support. Immobilization of the polypeptide may occur through an immobilized first antibody specific for the polypeptide. The immobilized first antibody is incubated with a sample suspected of containing the polypeptide. If present, the polypeptide binds to the first antibody.

A second antibody, also specific for the polypeptide, binds to the immobilized polypeptide. The second antibody may be labeled by methods known in the art. Non-immobilized materials are washed away, and the presence of immobilized label

indicates the presence of the polypeptide. This and other immunoassays are described by David, et al., in U.S. Patent 4,376,110 assigned to Hybritech, Inc., La Jolla, California.

The probe may also be a nucleic acid molecule that recognizes a FrpB nucleic acid molecule of the invention. Methods for determining whether a nucleic acid molecule probe recognizes a specific nucleic acid molecule in a sample are known in the art. Generally, a labeled probe that is complementary to a nucleic acid sequence suspected of being in a sample is prepared. The presence of probe hybridized to the target nucleic acid molecule indicates the presence of the nucleic acid molecule. Suitable methods are described by Schneider et al in U.S. Patent 4,882,269, which is assigned to Princeton University, and by Segev in PCT Application WO 90/01069, which is assigned to ImClone Systems Incorporated.

The probes described above are labeled in accordance with methods known in the art. Methods for labeling antibodies have been described, for example, by Hunter and Greenwood (93) and by David et al. (94). Additional methods for labeling antibodies have been described in U.S. patents 3,940,475 and 3,645,090. Methods for labeling oligonucleotide probes have been described, for example, by Leary et al (95); Renz and Kurz (96); Richardson and Gumport (97); Smith et al. (98); and Meinkoth and Wahl (99).

The label may be radioactive. Some examples of useful radioactive labels include ^{32}P, ^{125}I, ^{131}I, and ^{3}H. Use of radioactive labels have been described in U.K. 2,034,323, U.S. 4,358,535, and U.S. 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophors, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Some useful enzymatic labels include enzymes that cause a detectable change in a substrate. Some useful enzymes and their substrates include, for example, horseradish peroxidase (pyrogallol and o-phenylenediamine), beta-galactosidase (fluorescein beta-D-galactopyranoside), and alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The use of enzymatic labels have been described in U.K. 2,019,404, EP 63,879, and by Rotman (100).

Useful chromophores include, for example, fluorescent, chemiluminescent, and bioluminescent molecules, as well as dyes. Some specific chromophores useful in the present invention include, for example, fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, and luminol.

The labels may be conjugated to the antibody or nucleotide probe by methods that are well known in the art. The labels may be directly attached through a functional group on the probe. The probe either contains or can be caused to contain such a functional group. Some examples of suitable functional groups include, for example, amino, carboxyl, sulfhydryl, maleimide, isocyanate, isothiocyanate.

The label may also be conjugated to the probe by means of a ligand attached to the probe by a method described above and a receptor for that ligand attached to the label. Any of the known ligand-receptor combinations is suitable. The biotin-avidin combination is preferred.

The polypeptide of the invention may be used to detect the presence of antibodies specific for *N. gonorrhoeae* or *N. meningitidis* in a sample. The method comprises preparing a polypeptide containing a segment having an amino acid sequence that is substantially the same as a FrpB from either *N. gonorrhoeae* to detect antibodies to *N. gonorrhoeae* or *N. meningitidis* to detect antibodies to *N. meningitidis*. The polypeptide may be prepared as described above.

The sample may, for example, be from a patient suspected of being infected with *N. gonorrhoeae or N. meningitidis*. Suitable assays are known in the art, such as the standard ELISA protocol described by R.H. Kenneth (101).

Briefly, plates are coated with antigenic polypeptide at a concentration sufficient to bind detectable amounts of the antibody. After incubating the plates with the polypeptide, the plates are blocked with a suitable blocking agent, such as, for example, 10% normal goat serum. The sample, such as patient sera, is added and titered to determine the endpoint. Positive and negative controls are added simultaneously to quantitate the amount of relevant antibody present in the unknown samples. Following incubation, the samples are probed with goat anti-human Ig conjugated to a suitable enzyme. The presence of anti-polypeptide antibodies in the sample is indicated by the presence of the enzyme.

The following Examples section is set forth to aid in an understanding of the invention. This section is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXAMPLES

Strains and growth conditions. Bacterial strains used in this experiment are described in Table 1. *Neisseria* strains were routinely cultured on GCB media (Difco Laboratories) containing Kellogg's supplements I and II (29) and grown overnight at 35°C in an atmosphere of 5%CO_2. Antibiotic selection employed chloramphenicol at 1µg/ml for mTn3(Cm)(51) mutagenized strains and streptomycin at 100µg/ml for Ω (44) mutagenized strains.

For western blot analysis of total membrane proteins of iron-stressed gonococci, cells

were grown in CDM as previously described (13). Cultures were made iron replete as indicated by the addition of 100uM ferric citrate.

E.coli strains were routinely cultured on Luria-Bertani (LB) media (47). Antibiotic selection was 100μg/ml ampicillin, 100μg/ml streptomycin, 40μg/ml kanamycin, and/or 30μg/ml cholramphenicol. δ-aminolevulinic acid was used at 30μg/ml and heme at 50μg/ml. *E.coli* cultures were iron stressed by the addition of 200μM 2,2-diyridyl (Sigma Chemical Co., St. Louis, MO). Deferoxamine mesylate (desferal) was obtained from Ciba-Geigy (Basel, Switzerland).

SDS-PAGE and Western Blotting. SDS-PAGE was performed in 7.5% polyacrylamide resolving gel and 4.5% polyacrylamide stacking gel. Electrophoresis was carried out at either 40 mA for one gel, or 80 mA for two gels in the discontinuous buffer system of Laemmli (32). Transfer and development were as described previously (23,61).

Preparation of polyclonal antisera and monoclonal antibodies. Preparation of polyclonal antisera was described previously (8). Anti-FrpB monoclonal antibodies were generated by methods described previously (60).

DNA isolation, digestion, and Southern blot analysis. Chromosomal DNA was purified by CsC1-gradient centrifugation according to the methods of Stern et al. (54). Plasmids were purified by either CsC1 centrifugation or according to the instructions provided in the Magic Miniprep™ DNA Purification Kit (Promega; Madison WI). Southern blotting and DNA hybridizations were performed as previously described (13). Restriction enzymes, Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD) and were used according to the manufacturer's

specifications. λ-ZapII and pBluescript II SK+ were obtained from Stratagene (La Jolla, CA).

DNA sequencing and sequence analysis. CsCl-purified pUNCH319 and pUNCH325 were used as templates for double-stranded DNA sequencing (31) using United States Biochemical Sequenase and the dideoxy chain termination procedure of Sanger et al. (48). Both dG- and dI- labeling reactions were carried out for all primers. Both strands of pUNCH319 were sequenced using vector-specific or insert-specific primers. Exonuclease III/Exo VII nested deletions (40) were generated from the *Mlu* end of pUNCH325 and vector-specific primers were used to sequence individual deletion clones. Internal primers were used to sequence gaps between clones as well as the opposite strand. DNA sequences were analyzed with the Genetics Computer Group software package (15) (University of Wisconsin).

Mutagenesis and gonococcal transformation. pHP45Ω (44) was used to insertionally inactivate *frpB*. pUNCH321 was digested with *Bgl* I and ends were repaired with Klenow. pHP45Ω was digested with *Sma* I and the 2.0kb Ω fragment was isolated from an agarose gel according to the instructions provided in the Geneclean II Kit (Bio101 Inc. La Jolla, CA). Transformation of plasmid DNA into FA19 was as previously described (7).

Preparation of FrpB for amino-terminal sequence analysis. N-lauroylsarcosine (Sigma) insoluble membrane fractions were prepared from iron-stressed gonococcal strain UU1008 and protein concentration was determined by a bicinchoninic acid assay (BCA) (Pierce, Rockford, IL). Two hundred micrograms of protein was loaded into a preparative well of a 7.5% SDS-polyacryamide gel, poured 24 hours previously to permit TEMED (N,N,N',N'-tetramethylethylenediamine) and APS (ammonium persulfate) to evaporate. Electrophoresis was carried out at 40 mA constant current

using the discontinuous buffer system of Laemmli (32). The gel was soaked for 15 minutes in transfer buffer (13) before transferring. PVDF (polyvinylidene difluoride) membrane was placed in 100% methanol for two seconds, transferred to distilled deionized water (ddH_2O) for five minutes, and soaked in transfer buffer for 10 minutes prior to transfer. Transfer was for three and a half hours at 90mA in a submerged trans-blot apparatus (BioRad, Richmond, CA). Subsequent to transfer, the PVDF membrane was stained for five minutes in 0.1% Coomassie Brilliant Blue, 20% methanol, and 10% acetic acid to visualize proteins and destained for 10 minutes in ddH2O with one change. Filter was frozen at -20°C overnight. FrpB was identified by molecular weight and the amino-terminal amino acid sequence of the protein on the filter was determined by the Protein Microsequencing Facility at UCLA.

^{55}Fe uptake assays. Data were compiled from three individual experiments performed in triplicate on separate days. Gonococci were iron stressed as previously reported (2) prior to experimentation. SDS-PAGE and Western blotting of whole-cell lysates were routinely performed to determine that cultures were consistently and equivalently iron stressed, as evidenced by reactivity with anti-FrpB monoclonal antibody and/or anti-Tbp1 antisera. Iron-uptake assays were performed as previously reported (9) with the following modifications. Filters were blocked just prior to experimentation with 30µl. 10mg/ml BSA in 1XCDM. Assays were performed in 200µl volumes in 96 well filtration plates (MAHV Millipore, Bedford, MA) at 35°C in a 5% CO_2 atmosphere. Potassium cyanide was dissolved in 1XCDM. The vacuum manifold was from Millipore Multiscreen Assay System. Heme was used at 0.5µM, transferrin at 6.25µM, and citrate at 100µM. Membranes were air dried overnight, and the Millipore punch kit was used to separate and collect individual filters prior to counting. Data were expressed as counts per minute per µg of protein.

Preparation of aerobactin and enterobactin. Purified aerobactin and enterobactin

were the generous gift of P.E. Klebba. Aerobactin was ferrated as follows. Ferric sulfate was dissolved to 4mM in 50ml ddH_2O containing 1.5µl HCl. 400µ 4mM aerobactin was added to 400µl 4mM ferric sulfate and 80µl 0.5M Na_2HPO_4 The ferri-aerobactin was run over a CM-cellulose (Sigma, St. Louis, MO) column equilibrated in 0.05M Na_2HPO_4. The final concentration of aerobactin was determined by reading the absorbance at 400nM (24).

Iron sources. Human transferrin, human lactoferrin, bovine heme, human hemoglobin, and human haptoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). ^{55}Fe hemin was purchased from the custom synthesizing facility at NEN Products Dupont (Wilmington, DE) lot number FE55.1193RS. Transferrin, lactoferrin, and citrate were ferrated with $^{55}FeC1$ as previously described (36).

RNase assay. The RNase assay was performed as previously described (71), except 0.1N HCl was used instead of 0.5N HCl.

Hemin affinity purification. Hemin agarose was purchased from Sigma Chemical Co. (St. Louis, MO). The method of affinity purification was described by Lee (33).

Bactericidal assays. Bactericidal assays were performed as described previously (18).

Cloning the gonococcal frpB gene. Sarcosyl insoluble membrane fractions from gonococcal strain UU1008 were used to obtain FrpB N-terminal amino acid sequence (see above). A degenerate oligonucleotide containing inosine (designated MB.3, shown in Fig. 1) was deduced from this sequence and used to probe a Southern blot of FA19 chromosomal DNA. Each restriction digest contained a single hybridizing band. A 5.8kb *Dra* I fragment was chosen for further analysis.

A λ-ZapII library containing *Eco*RI-linkered FA19 chromosomal *Dra* I fragments (2) was screened with oligo MB.3. Approximately one positive plaque was identified for every 10,000 plaques screened. Attempts to excise the phagemid containing the intact insert consistently resulted in deletion products smaller than pBluescript II SK* alone. Since such a large chromosomal fragment potentially contained both the *frpB* promoter and entire f*rpB* coding sequence and that the expression of FrpB might be toxic in *E.coli*, smaller fragments were subcloned into pBluescript II SK*.

DNA prepared from one of the positively hybridized plaques, λfrpB-4(Fig. 2), was digested with *Eco*RI to release the insert DNA. The expected 5.8kb fragment was isolated from an agarose gel and further digested with *Cla* I to generate a 540bp, MB.3-hybridizing fragment and an approximately 5.3kb fragment which did not hybridize to MB.3. The smaller fragment ligated into pBluescript II SK* was stable in *E.coli* DH5αMCR and was designated pUNCH319. The larger fragment ligated into pBluescript II SK* generated pUNCH320. pUNCH320 caused *E.coli* DH5αMCR to grow poorly and appeared to be severely restricted in copy number. These data suggested that other sequences located 3' of *frpB* may also be toxic to *E.coli* and that further subcloning was necessary to obtain stable clones. Digestion of pUNCH320 with *Mlu* I and *Eco*R I released fragments of approximately 1.0 kb and 1.5kb, leaving a 2.8kb *Cla* I-*Mlu* I fragment attached to pBluescript II SK*. This 5.8kb fragment (vector plus 2.8kb *Cla* I-*Mlu* I insert) was subsequently isolated, treated with Klenow, and re-ligated to itself to generate pUNCH325. DH5αMCR (pUNCH325) transformants were stable and the plasmid copy number apparently normal.

Nucleotide sequence and analysis of *frpB*. PCR amplification of chromosomal DNA followed by sequence analysis of clones confirmed the *Cla* I junction between pUNCH319 and pUNCH325. The combined nucleotide sequence and deduced amino

acid sequence from pUNCH319 and pUNCH325 are shown in Fig. 3. Putative promoter sequences were located upstream of a well conserved Fur box (4). A string of nine cytosine residues was noted between the putative -10 and -35 RNA-polymerase binding sites. A Shine-Dalgarno sequence starting at nucleotide 307 and ending at nucleotide 310 (Fig. 3), was located six bases before an ATG codon, the start of a 1,925bp open reading frame (ORF). This ORF encoded a protein of 713 amino acids. The predicted protein contained a typical signal sequence and characteristic Ala-X-Ala. signal peptidase I cleavage site. The first ten amino acids adjacent to the cleavage site were identical to the peptide sequence obtained from the mature FrpB protein. A classical TonB box was noted at residues 32-36. The mature protein had a calculated molecular weight of 76.6 kD and an isoeletric point of 10.38. The sequence downstream of the ORF revealed an inverted repeat but no string of T residues characteristic of rho-independent transcription termination (69). The protein terminated with an aromatic residue preceded by nine alternating hydrophobic and hydrophilic amino acids. This structure is typical of many bacterial outer membrane proteins sequenced to date (58).

GenBank homologies. Comparison of FrpB with other sequences in GenBank revealed some interesting homologies. Several regions of the predicted FrpB protein shared similarity with regions identified in other proteins as potentially important for membrane localization and/or TonB interaction. Localized homology was found between FrpB and the family of TonB-dependent outer membrane receptor proteins including BtuB (25) and FepA (35) of *E.coli* and between Tbp1 (13) and IroA (42) of *Neisseria* species. This similarity was limited to the highly conserved domains (13), and suggested that FrpB may also be a TonB-dependent receptor. More similarity was found with HemR, the hemin receptor of *Yersinia enterocolitica* (55). HemR is an iron-regulated, outer membrane protein that is also a member of the family of TonB-dependent receptor proteins. Overall the two proteins were 26% identical and 48% similar. The most notable similarity was seen with CopB, a major outer membrane

protein of *Moraxella catarrhalis* (26). Overall FrpB and CopB were 52% identical and 71% similar.

Transposon mutagenesis of *frpB*. In order to construct FrpB mutants, the gonococcal insert in pUNCH319 was ligated into pUP1(19), creating pUNCH321. The Ω fragment from pHP45Ω was ligated into a unique *Bgl* I site in pUNCH321 (Insertion site shown in Fig. 3). This DNA was reintroduced into the chromosome of gonococcal strain FA19 by transformation and allelic replacement, creating FA6807. Southern blot analysis of chromosomal DNA from FA19 and FA6807 indicated that a 450bp. MB.3-hybridizing, *Hin*cII fragment present in the parent was missing in FA6807 and a new reactive band of approximately 2.5kb was present (Fig. 4, panel A). An identical blot (Fig 4, panel B) probed with Ω, only hybridized to the 2.5kb fragment in FA6807. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with anti-FrpB monoclonal antibody W.6, confirmed that FrpB was absent from this strain (Fig. 5).

The Ω insertion in *frpB* was also introduced into FA6747 (*tbpA*::mTn3(Cm)) by transformation and allelic replacement creating FA6808. The FrpB$^-$/Tbp1$^-$ phenotype of FA6808 was confirmed by SDS-PAGE and Western blot analysis. This strain was used for FrpB function analysis as described below.

Utilization of iron sources. In an attempt to determine the function that FrpB plays in iron utilization, FA19 and FA6807 were grown in chemically-defined media (CDM) lacking iron. Aliquots of iron-stressed cultures were plated onto CDM agarose containing 10μM Desferal and GC base agar containing 50μM Desferal. Sterile 3mm discs containing either citrate, transferrin, lactoferrin, heme, hemoglobin, or hemoglobin bound to haptoglobin were positioned around each plate. One disc without any added iron source was added as a negative control. After overnight incubation, growth of both

strains was evident around all discs except the negative control.

N. gonorrhoeae can utilize aerobactin (67) and enterobactin (45) as iron sources. To determine if FrpB functioned as either an aerobactin or enterobactin receptor, FA19, FA6808, FA6747, KDF541, KDF541/pABN6, and BN1071 (Table 1) were iron stressed in CDM as above and plated onto CDM agarose containing 2.5μM 30% iron-saturated transferrin. FA6747 and FA6808 could not use Tf as an iron source because they lacked Tbp1, therefore these strains could grow only in the presence of a functional high-affinity siderophore receptor. Three sterile discs were positioned around each plate. Either 30% saturated lactoferrin (positive control for gonococcal viability) or filter-sterilized, iron-free supernatant from LG1315 pColV (aerobactin producer) or AN102 (enterobactin hyper-producer) were added to each disk. After overnight incubation, *E.coli* controls grew as expected suggesting that both siderophores were efficient at stripping iron from transferrin, the sole iron source provided in the media. FA19 grew over the entire transferrin plate as expected, however, growth of FA6808 and FA6747 was only evident around the lactoferrin disks, suggesting that the cells were viable but unable to use aerobactin or enterobactin under these conditions.

Aerobactin utilization by FA19 and FA6807 was further evaluated in chemically-defined, liquid media, employing various concentrations of purified ferri-aerobactin (Fig. 6). The aerobactin receptor-negative *E.coli* strain KDF541 and aerobactin receptor-positive *E.coli* strain KDF541(pABN6) were used as controls. These data suggested that *N. gonorrhoeae* FA19 and FA6807 used ferri-aerobactin similarly and in a concentration-dependent fashion analogous to the aerobactin receptor-negative *E.coli* control. Growth stimulation of gonococci by ferri-aerobactin required relatively high concentrations (3μM) and never attained a density equivalent to that of the Tf or citrate controls. These experiments confirmed the ability of gonococci to utilize ferri-aerobactin as an iron source *in vitro* but showed that this ability was not dependent upon a high-affinity receptor-mediated event.

^{55}F uptake from hemin, Tf, and citrate. Because of the high degree of similarity between HemR, a known hemin receptor in *Y.enterocolitica* and FrpB, it was analyzed whether a quantitative difference in ^{55}Fe uptake from hemin could be detected between FA19 and FA6807. Uptake of ^{55}Fe from transferrin by FA19, FA6807, and the Tbp1 mutant FA6747 were used as controls. The results indicated that while ^{55}Fe uptake from transferrin was approximately wild type in FA6807(P=.826), ^{55}Fe uptake from hemin was reduced by approximately 60% (P<0.001)(Fig. 7). Surprisingly, ^{55}Fe uptake from hemin was also significantly reduced in FA6747 (P<0.001). To determine whether the inability to use ^{55}Fe from hemin was specific to FA6807(FrpB$^-$) and FA6747 (Tbp1$^-$), ^{55}Fe uptake from hemin was assayed in other well-characterized, gonococcal mutants specifically altered in the expression of other iron-repressible proteins. The Tbp2$^-$ and Lbp$^-$ strains, FA6819 and FA6775 respectively, were also reduced in ^{55}Fe internalization from hemin (P<0.001). These data suggested that either more than one protein was involved in the internalization of hemin iron or the notable decrease in hemin-iron uptake in these mutants resulted from unanticipated, non-specific effects of each of these mutations on a separate membrane-bound, heme-iron-uptake system.

Reconstruction of *frpB* in pACYC184 and functional complementation of RK1065(*hemA*). In an attempt to determine if FrpB could function as a heme receptor, an *E.coli hemA* mutant was complemented with FrpB. Although expression of FrpB from the high copy-number vector pBluescript II SK$^-$ was toxic to *E.coli*, expression from the low copy-number vector pACYC184 was tolerated. The *frpB* reconstruction strategy is outlined in Fig. 8. Briefly, the insert from pUNCH319 was ligated into the *Cla* I and *Bam*H I sites of pACYC184, generating pUNCH330. pUNCH330 was digested with *Cla* I and the gel-purified *Cla* I-*Xba* I fragment from pUNCH325 was ligated into this site as follows. After ligating for four hours, Klenow was added to the ligation mixture for 30 minutes at room temperature to repair non-ligated *Cla* I and *Xba*

I ends. The reaction was further ligated overnight. The *frpB* clone in pACYC184 was designated pUNCH331. FrpB expression from pUNCH331 was iron repressible, suggesting regulation by *E.coli* Fur.

RK1065 is an *E.coli hemA* mutant which is unable to synthesize or internalize heme (27). Growth stimulation requires either δ-aminolevulinic acid, or heme and a functional heme receptor. Transformation of pUNCH331 into RK1065 supported growth on heme plates, whereas pACYC184 alone did not (Fig. 9). An Rnase leakage assay was performed to determine if FrpB expression altered the *E.coli* outer membrane, thereby allowing heme to simply diffuse into the cell (71). The *E.coli* strains C386 and HB101 containing pEBH21 were used as positive and negative controls respectively. No difference in leakiness was detected between RK1065 (pACYC184) and RK1065 (pUNCH331), suggesting that growth of RK1065 (pUNCH331) on heme plates was not due to a membrane perturbation gross enough to permit leakage of the periplasmic protein RNase H. Nevertheless, RK1065 (pUNCH331) was more sensitive to several hydrophobic antibiotics than the same strain with pACYC194 alone (Fig. 9). This experiment suggested that the presence of FrpB in *E.coli* probably allowed heme to enter non-specifically either by creating a pore or by perturbing the integrity of the outer membrane. Uptake of ^{55}Fe from hemin in RK1065 (pUNCH331) was not inhibited by KCN, consistent with a non-specific, non-receptor mediated mechanism of uptake.

Bactericidal Assay. In *M. catarrhalis*, CopB, the protein with the greatest similarity to FrpB, appears to play a major role in serum resistance. Mutants which are missing CopB have decreased serum resistance. Mutants which are missing CopB have decreased serum resistance and survival in a mouse model (26). Standard bactericidal assays were performed with normal human serum on FA19 and FA6807 grown under iron-limiting conditions and were unable to detect any difference in survival; both strains were completely serum resistant.

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Table 1. Bacterial strains, plasmids and phage.

Strain, plasmid or phage	Description	Source/reference
FA19	Wild type	[Mickelsen, 1981 #38]
FA6807	*frpB*::Ω(FrpB$^-$)	This study
FA6808	*frpB*::Ω tbpA::mTn3(Cm) (FrpB$^-$, Tbp1$^-$)	This study
FA6747	*tbpA*::mTn3(Cm) (Tbp1$^-$)	[Cornelissen, 1992 #13]
FA6819	Δ*tbpB* (Tbp2$^-$)	[Anderson, 1994 #2]
FA6775	*lbpA*::mTn3(Cm) (Lbp$^-$)	[Biswas, 1994 #6]
UU1008	Wild type	Zell McGee
DH5αMCR	*F$^-$ mcrA mcrB mrr* φ80*dlacZ*ΔM15 Δ(*argF-lac*)*U169 recA1 endA1 hsdR hsdM supE44* λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Labs
BN1071	*F$^-$, pro, trp, rslL, entA* (Ent$^-$, FepA$^+$)	[Klebba, 1982 #30]
AN102	BN1071, *leu, fepA* (Ent$^+$, FepA$^-$)	[Klebba, 1982 #30]
KDF541	BN1071, *entA, fepA* (Ent$^-$, FepA$^-$)	[Rutz, 1992 #46]
KDF541 / pABN6	(Ent$^-$, FepA$^-$,lutA$^+$, luc$^-$)	[de Lorenzo, 1987]
LG1315/ pcolV	BN1071, *cir* (lutA$^+$, luc$^+$)	[Warner, 1981 #63]
RK1065	*hemA*	R. Kadner
HB101	*F$^-$, hsd20 (r_B^-,m_B^-), recA13, ara-14, proA2, lacYl, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44,* λ$^-$	Maniatis et. al. 1982
C386	ompA lpp	[Sonntag, 1978 #53]
pACYC184	ori p15a, Cm^R, Tc^R	New England Biolabs
pBluescript II SK+	ori pMB1, Ap^R	Stragene
pHP45Ω	source for the Ω fragment (Sm^R)	[Prentki, 1984 #44]
pUP1	pHSS6 containing gonococcal uptake seqeunce (Kan^R)	[Elkins, 1991 #19]

pEBH21	pBC II SK$^+$ derivative (Cm^R)	[Hardham, 1994 #22]
pUNCH319	pBluescript II SK$^+$ containing 540bp *Eco*R I-*Cla* I fragment from λfrpB.4	This Study
pUNCH320	pBluescript II SK$^+$ containing 5.3kb *Cla* I-*Eco*RI fragment from λfrpB.4	This Study
pUNCH321	pUP1 containing 540bp *EcoR* I-*Cla* fragment from pUNCH319	This Study
pUNCH324	pUNCH321 containing Ω fragment from pHP45Ω in unique *Bgl* I site	This Study
pUNCH325	pBluescript II SK$^+$ containing 2.8kb *Cla* I-*Mlu* I fragment from pUNCH320	This Study
pUNCH330	540bp *EcoR* I-*Cla* fragment from pUNCH319 in pACYC184	This Study
pUNCH331	reassembled gonococcal *frpB* gene in pACYC184	This Study
λ ZapII	excisable lambda phage vector	Statagene

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CLAIMS

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein.

2. An isolated nucleic acid molecule of claim 1 comprising the nucleotide sequence of Figure 3.

3. An isolated nucleic acid molecule of claim 1 comprising the nucleotide sequence of Figure 10.

4. An isolated nucleic acid molecule of claim 1, wherein the FrpB protein is the FrpB protein of *Neisseria gonorrhoeae*.

5. An isolated nucleic acid molecule of claim 1, wherein the FrpB protein is the FrpB protein of *Neisseria meningitidis*.

6. A polypeptide encoded by the isolated nucleic acid molecule of claim 2.

7. A polypeptide encoded by the isolated nucleic acid molecule of claim 3.

8. A vector which comprises the nucleic acid molecule of claim 1.

9. A vector of claim 8, wherein the nucleic acid molecule is linked to a plasmid.

10. A host vector system for the production of a polypeptide having the biological activity of a FrpB antigenic polypeptide which comprises the vector of claim 8 in a

suitable host.

11. A host vector system of claim 10, wherein the suitable host is a bacterial cell or animal cell.

12. A method of producing a polypeptide having the biological activity of a FrpB antigenic polypeptide which comprises growing the host vector system of claim 10 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

13. A method of producing a vaccine composition that protects a mammal from infection by *N. gonorrhoeae* comprising combining the FrpB protein encoded by the isolated nucleic acid of claim 1 with a pharmaceutically acceptable carrier.

14. The method of claim 13 further comprising combining the FrpB with an effective amount of an adjuvant.

15. The method of claim 13, wherein the amino acid sequence of the polypeptide comprises the FrpB protein of *N. gonorrhoeae.*

16. The method of claim 13, wherein the mammal is a human.

17. A method of producing a vaccine composition that protects a mammal from infection by *N. meningitidis* comprising combining the FrpB protein encoded by the isolated nucleic acid of claim 1 with a pharmaceutically acceptable carrier.

18. The method of claim 17 further comprising combining the FrpB with an effective amount of an adjuvant.

19. The method of claim 17. wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. meningitidis*.

20. The method of claim 17. wherein the mammal is a human.

21. A vaccine composition capable of protecting a mammal against infection by *N. gonorrhoeae*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

22. The vaccine composition of claim 21 further comprising an effective amount of an adjuvant.

23. The vaccine composition of claim 21, wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. gonorrhoeae*.

24. The vaccine composition of claim 21, wherein the mammal is a human.

25. A vaccine composition capable of protecting a mammal against infection by *N. meningitidis*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

26. The vaccine composition of claim 25 further comprising an effective amount of an adjuvant.

27. The vaccine composition of claim 25, wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. meningitidis*.

28. The vaccine composition of claim 25, wherein the mammal is a human.

29. A method of protecting a mammal against infection by *N. gonorrhoeae* comprising administering to the mammal a vaccine composition of claim 21.

30. A method of protecting a mammal against infection by *N. meningitidis* comprising administering to the mammal a vaccine composition of claim 25.

31. An antibody directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of claim 2.

32. An antibody directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of claim 3.

33. A method of detecting an antibody specific for *N. gonorrhoeae* in a sample comprising:

(a) contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of claim 4 under conditions to form a complex between the polypeptide and the antibody; and

(b) detecting any complex so formed;

thereby detecting an antibody specific for *N. gonorrhoeae*.

34. A method of claim 33, wherein the FrpB protein is labeled with a detectable marker.

35. A method of detecting an antibody specific for *N. meningitidis* in a sample comprising:

(a) contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of claim 5 under conditions to form a complex between the polypeptide and the antibody; and

(b) detecting any complex so formed;

thereby detecting any antibody specific for *N. meningitidis*.

36. A method of claim 35. wherein the FrpB protein is labeled with a detectable marker.

37. A method of treating a mammal infected by *N. gonorrhoeae* comprising administering to the mammal an antibody of claim 31.

38. A method of treating a mammal infected by *N. gonorrhoeae* comprising administering to the mammal an antibody of claim 32.

39. The method of claim 37 or 38 wherein the mammal is a human.

40. The method of claim 37 or 38 wherein the antibody is monoclonal.

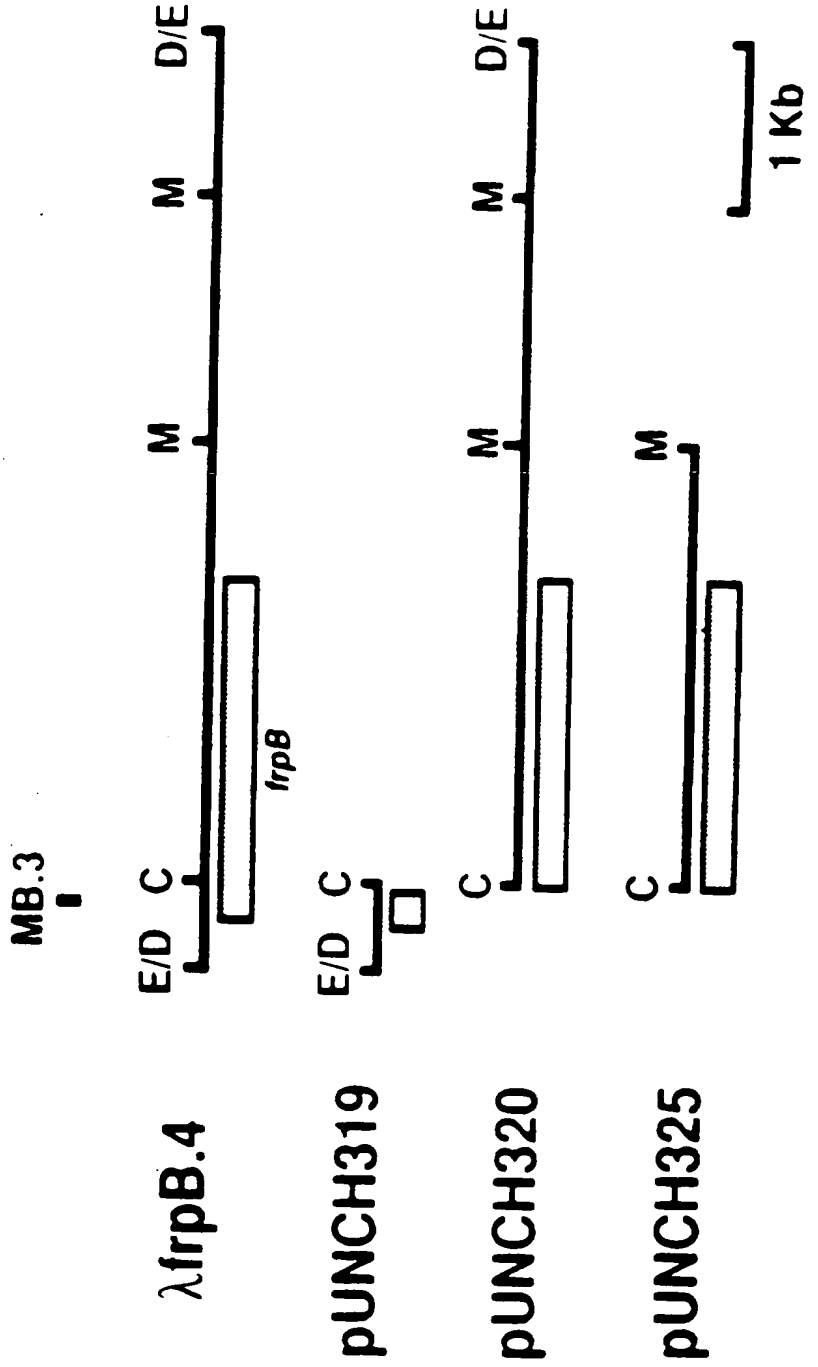
41. A method of treating a mammal infected by *N. meningitidis* comprising administering to the mammal an antibody of claim 31.

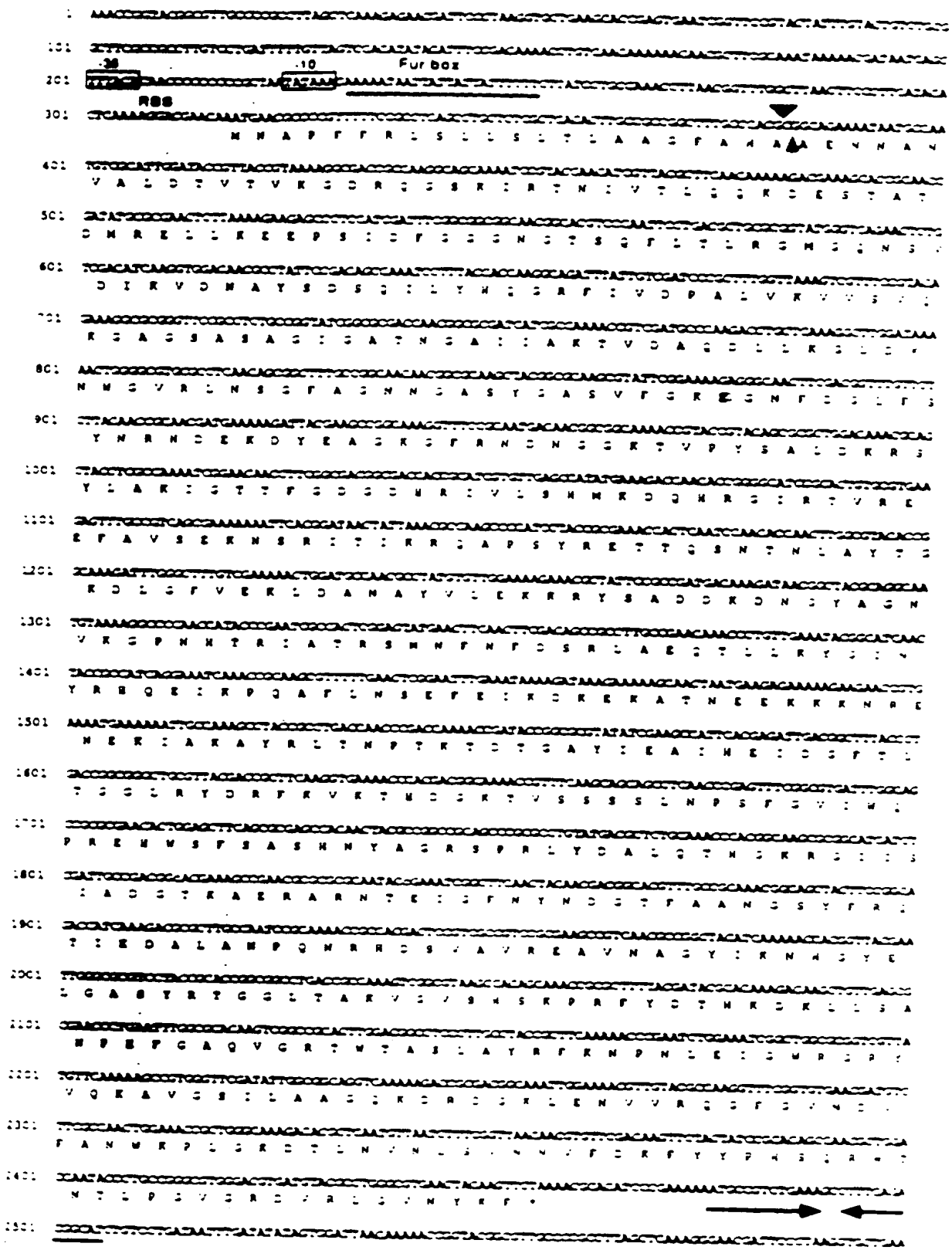
42. A method of treating a mammal infected by *N. meningitidis* comprising administering to the mammal an antibody of claim 32.

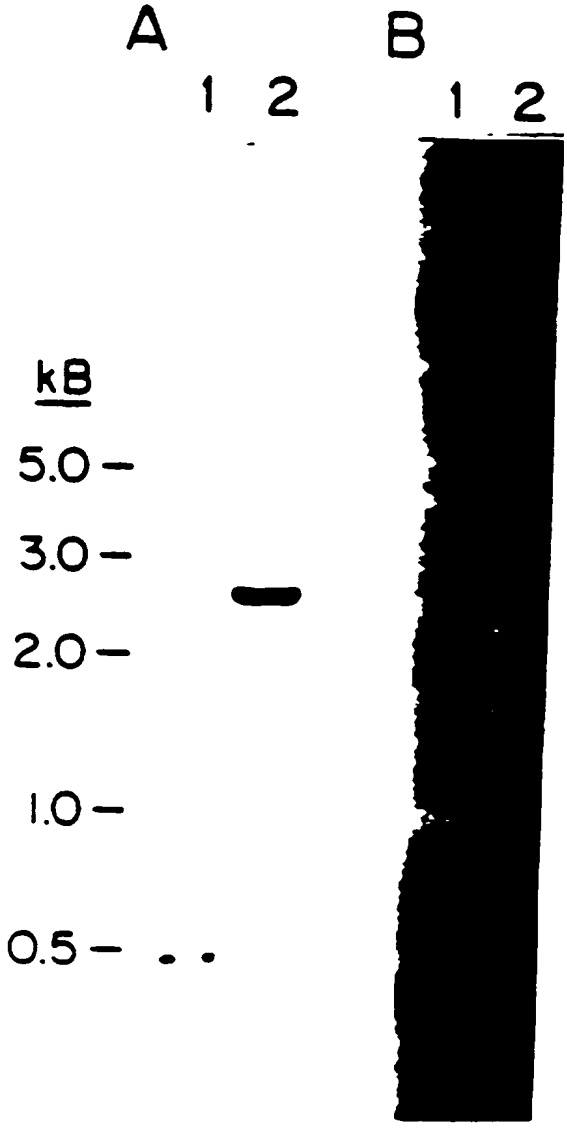
43. The method of claim 41 or 42 wherein the mammal is a human.

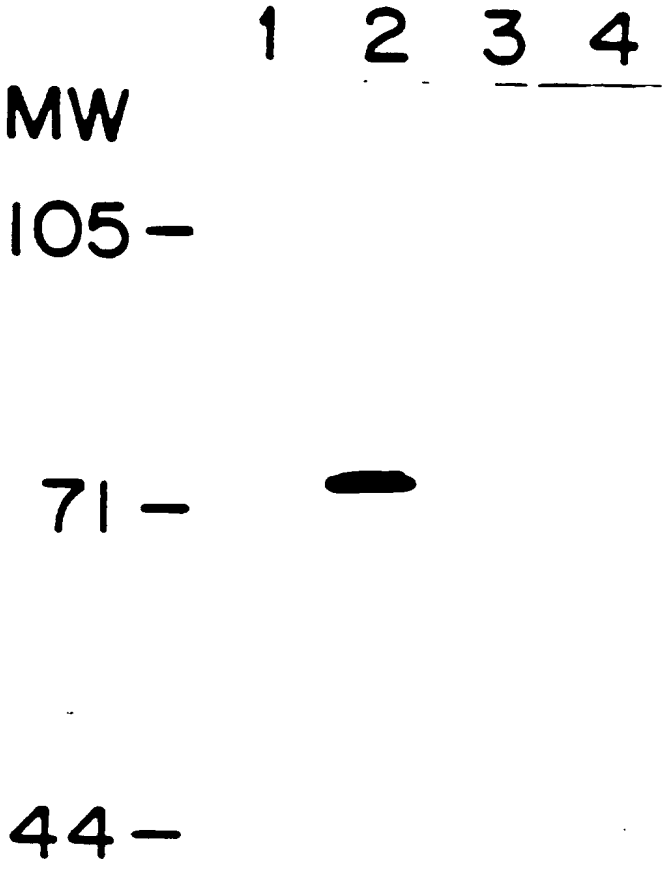
44. The method of claim 41 or 42 wherein the antibody is monoclonal.

FIGURE 1









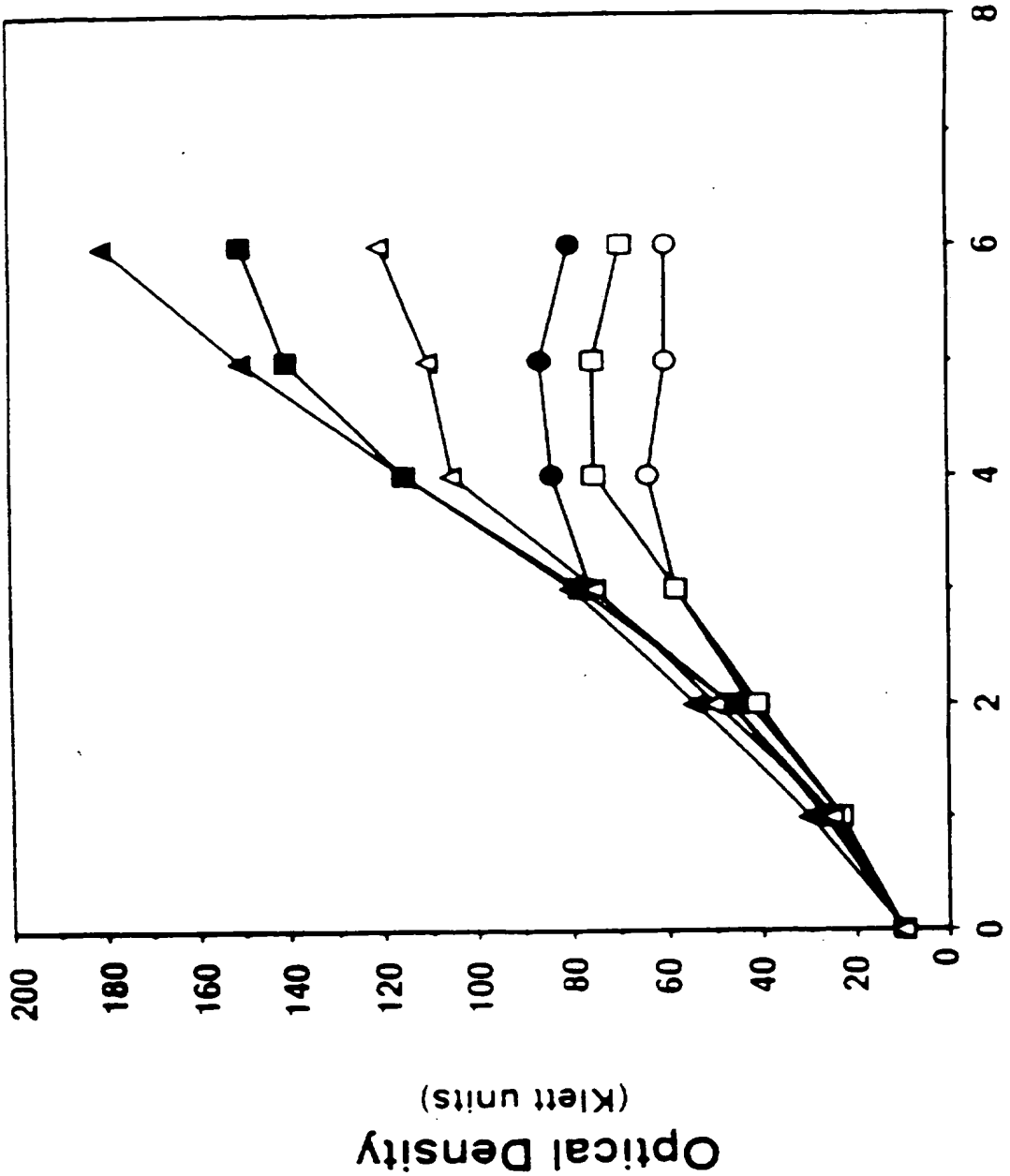
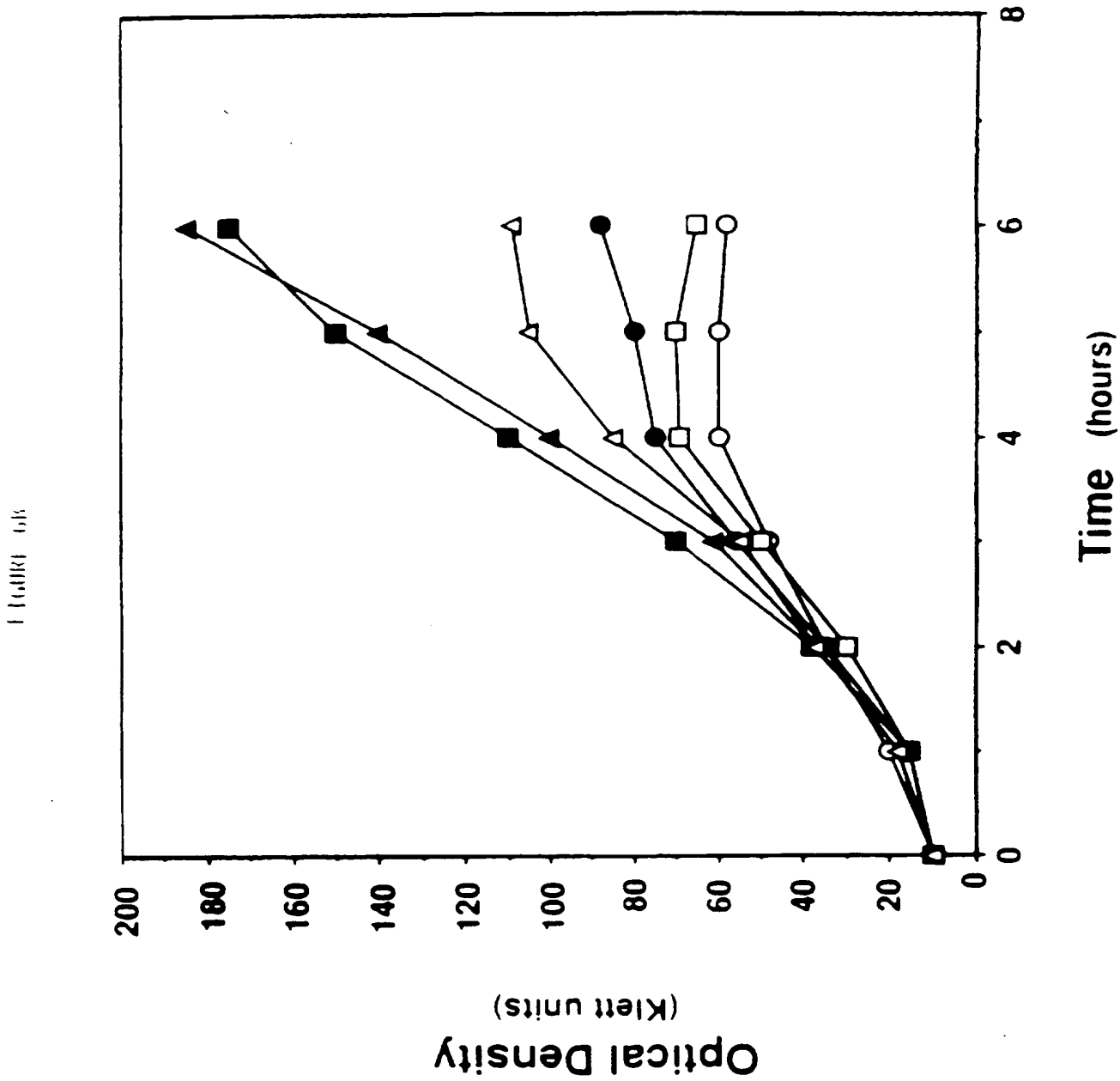


FIGURE 6A



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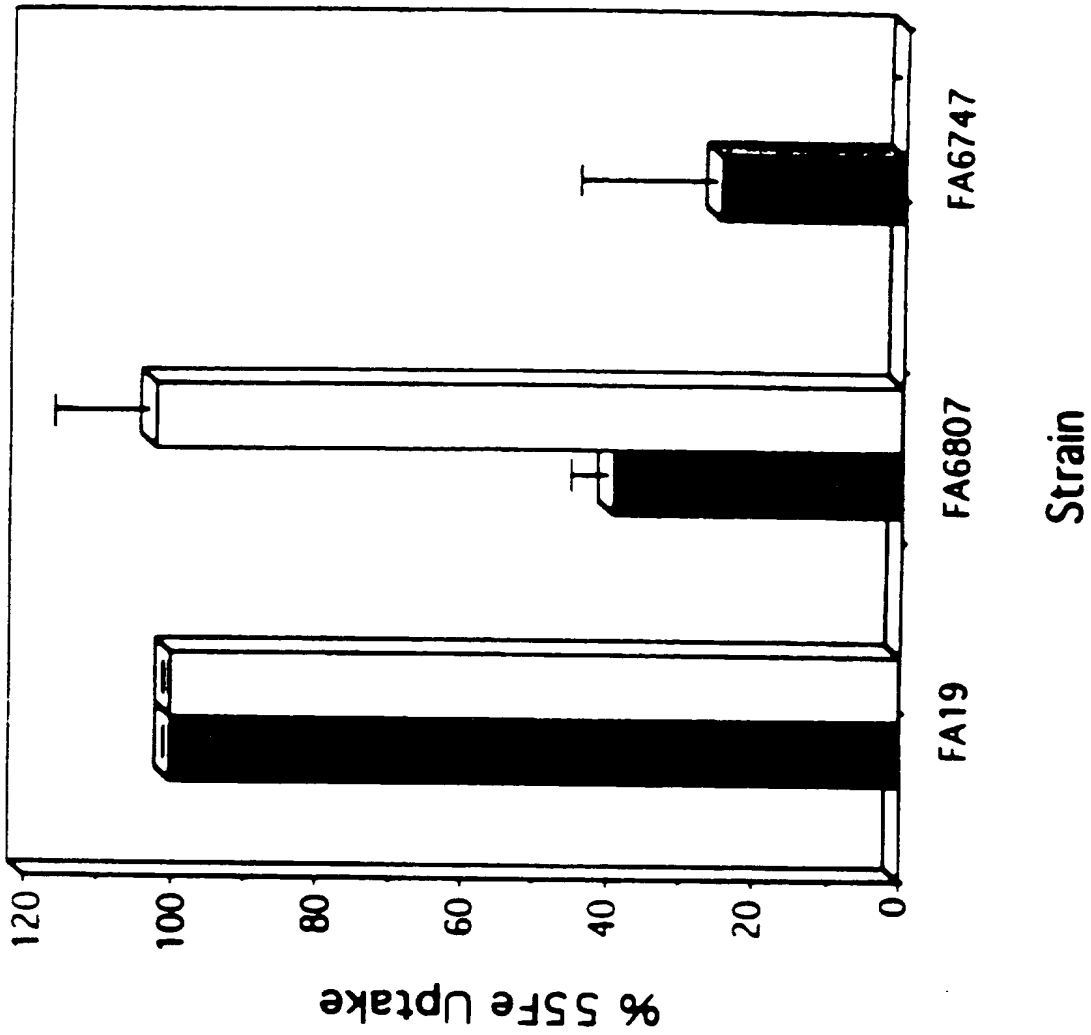
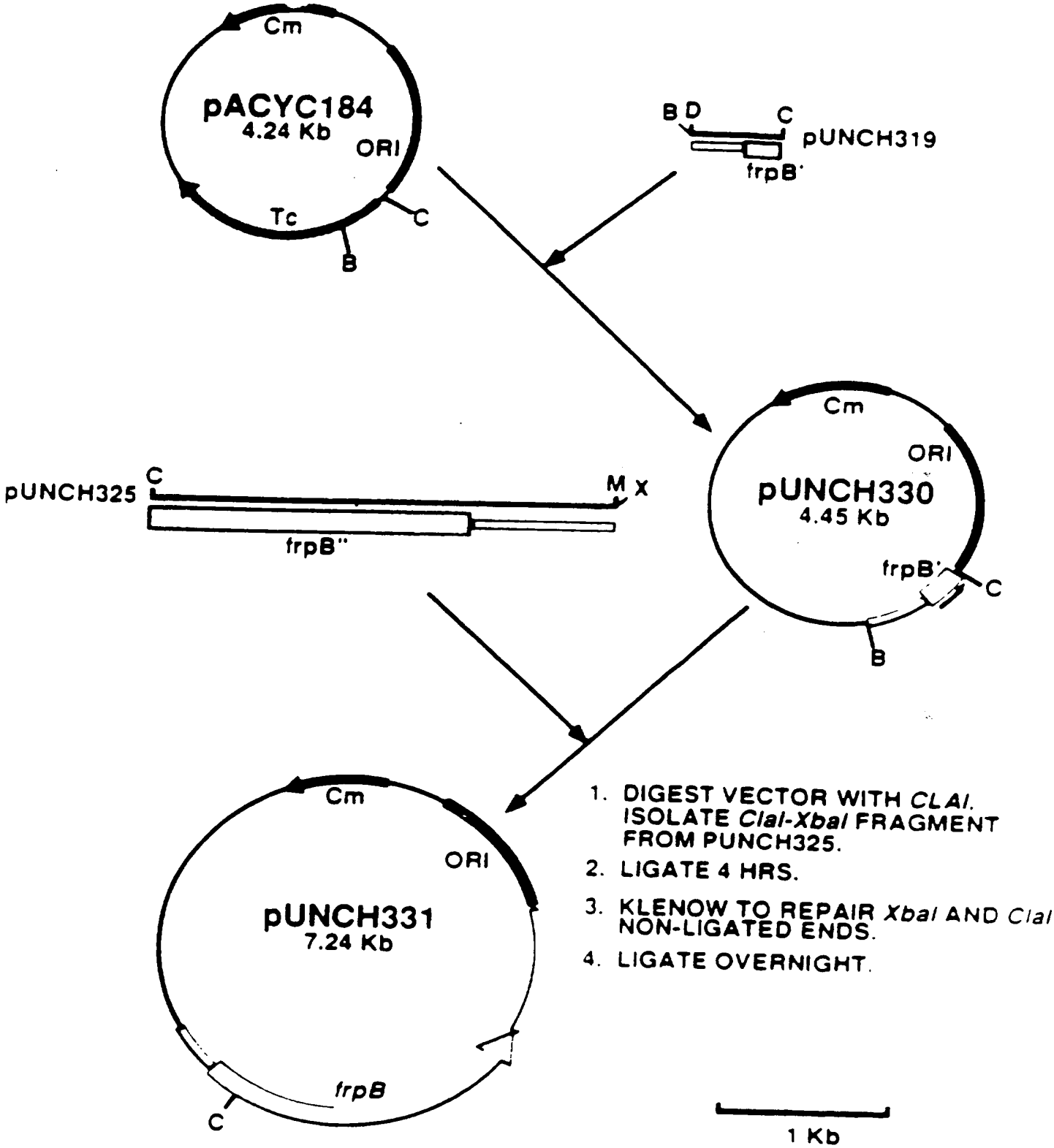
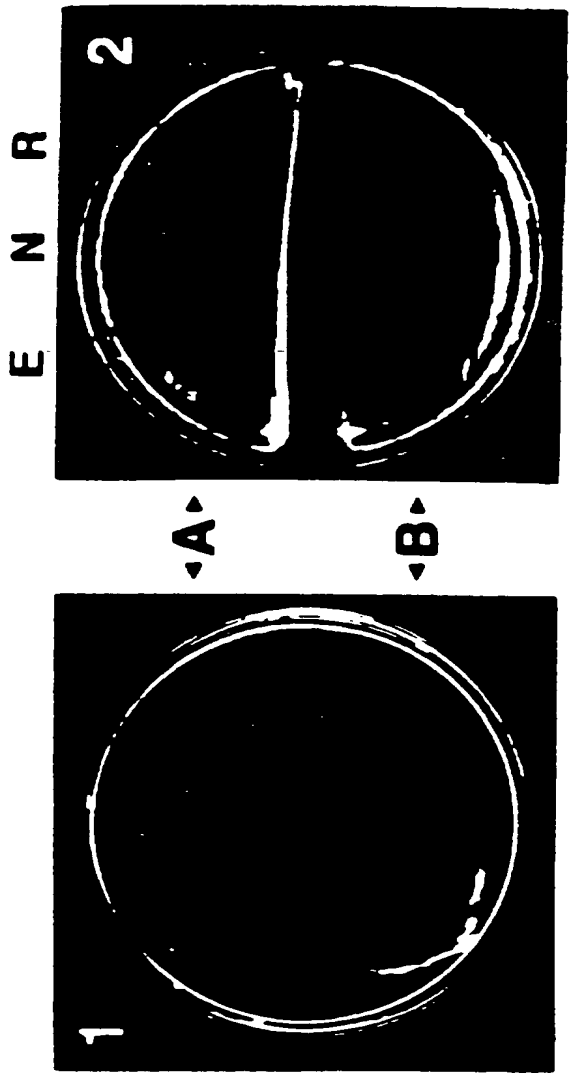


FIGURE 1





Sequence Range 1 to 2369

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          70         80         90        100        110        120
          .          .          .          .          .          .          .
GCTAATATAA ACAAAAATAA TTATTATTAT TTTTTCTTAT CCTGCCAAAC CTTAACGGTT

         130        140        150        160        170
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TGGCTTAACT TCCCTTCATA CACTCAAAAG GACGAACAA ATG AAC GCC CCG TTT TTC
                                             Met Asn Ala Pro Ph  Phe>
                                             ___TRANSLATION OF P____>

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Arg Leu Ser Leu Leu Ser Leu Thr Leu Ala Ala Gly Phe Ala His Ala>
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Asp Arg Gln Gly Ser Lys Ile Arg Thr Asn Ile Val Thr Leu Gln Gln>
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Pro Ser Ile Asp Phe Gly Gly Gly Asn Gly Thr Ser Gln Phe Leu Thr>
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420        430        440        450        460
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   470        480        490        500        510
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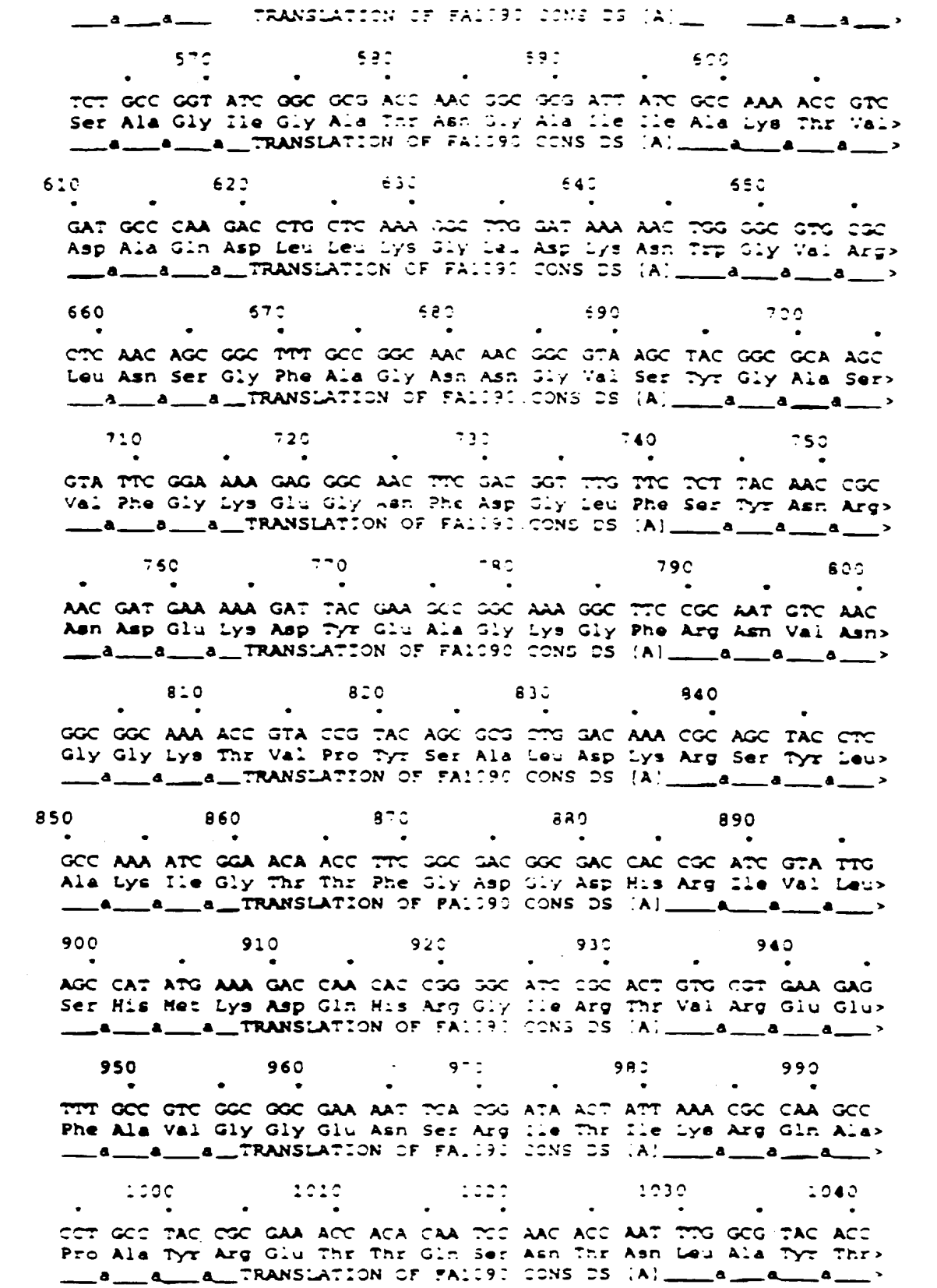

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Ser Gln Phe Lys Ile Glu Asp Lys Lys Asp Ala Thr Glu Glu Asp Lys>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

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 .           .           .           .
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___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1620 1630 1640 1650 1660

ACC CAC GGC AAA CGC GGC ATC ATC TCG ATT GCC GAC GGC ACC AAA GCC
Thr His Gly Lys Arg Gly Ile Ile Ser Ile Ala Asp Gly Thr Lys Ala>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1670 1680 1690 1700 1710

GAA CGC GCG CGC AAT ACC GAA ATC GGC TTC AAC TAC AAC GAC GGC ACG
Glu Arg Ala Arg Asn Thr Glu Ile Gly Phe Asn Tyr Asn Asp Gly Thr>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1720 1730 1740 1750 1760

TTT GCC GCA AAC GGC AGC TAC TTC CGG CAG ACC ATC AAA GAC GCG CTT
Phe Ala Ala Asn Gly Ser Tyr Phe Arg Gln Thr Ile Lys Asp Ala Leu>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1770 1780 1790 1800

GCC AAT CCG CAA AAC CGC CAC GAC TCT GTC GCC GTC CGC GAA GCC GTC
Ala Asn Pro Gln Asn Arg His Asp Ser Val Ala Val Arg Glu Ala Val>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1810 1820 1830 1840 1850

AAC GCC GGC TAC ATC AAA AAC CAC GGT TAC GAA TTG GGC GCG TCC TAC
Asn Ala Gly Tyr Ile Lys Asn His Gly Tyr Glu Leu Gly Ala Ser Tyr>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1860 1870 1880 1890 1900

CGC ACC GGC GGC CTG ACC GCC AAA GTC GGC GTA AGC CGC AGC AAA CCG
Arg Thr Gly Gly Leu Thr Ala Lys Val Gly Val Ser Arg Ser Lys Pro>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1910 1920 1930 1940 1950

CGC TTT TAC GAT ACC CAT CCT AAA AAA CTG TTG AGC GCG AAC CCC GAG
Arg Phe Tyr Asp Thr His Pro Lys Lys Leu Leu Ser Ala Asn Pro Glu>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

1960 1970 1980 1990 2000

TTT GGC GCA CAA ACC GGC CGC ACT TGG ACG GCC TCC CTT GCC TAC CGC
Phe Gly Ala Gln Thr Gly Arg Thr Trp Thr Ala Ser Leu Ala Tyr Arg>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2010 2020 2030 2040

TTC AAA AAC CCG AAT CTG GAA ATC GGC TGG CGC GGA CGC TAT GTT CAA
Phe Lys Asn Pro Asn Leu Glu Il Gly Trp Arg Gly Arg Tyr Val Gln>

```
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2050          2060          2070          2080          2090
 .     .       .     .       .     .       .     .       .     .
AAA GCT ACG GGT TCG ATA TTG GCG GCA GGG CAA AAA GAC CGC GAC GGC
Lys Ala Thr Gly Ser Ile Leu Ala Ala Gly Gln Lys Asp Arg Asp Gly>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2100          2110          2120          2130          2140
 .     .       .     .       .     .       .     .       .     .
AAA TTG GAA AAC GTT GTA CGC CAA GGT TTC GGT GTG AAC GAT GTC TTC
Lys Leu Glu Asn Val Val Arg Gln Gly Phe Gly Val Asn Asp Val Phe>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2150          2160          2170          2180          2190
 .     .       .     .       .     .       .     .       .     .
GCC AAC TGG AAA CCG CTG GGC AAA GAC ACG CTC AAT GTT AAT CTT TCG
Ala Asn Trp Lys Pro Leu Gly Lys Asp Thr Leu Asn Val Asn Leu Ser>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2200          2210          2220          2230          2240
 .     .       .     .       .     .       .     .       .     .
GTT AAC AAC GTG TTC GAC AAG TTC TAC TAT CCG CAC AGC CAA CGC TGG
Val Asn Asn Val Phe Asp Lys Phe Tyr Tyr Pro His Ser Gln Arg Trp>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2250          2260          2270          2280
 .     .       .     .       .     .       .     .       .     .
ACC AAT ACC CTG CCG GGC GTG GGA CGT GAT GTA CGC CTG GGC GTG AAC
Thr Asn Thr Leu Pro Gly Val Gly Arg Asp Val Arg Leu Gly Val Asn>
___a___a___a__TRANSLATION OF FA1090 CONS DS (A)____a___a___a___>

2290          2300          2310          2320          2330          2340
 .     .       .     .       .     .       .     .       .     .       .     .
TAC AAG TTC TAA AACGCACAT CCCGAAAAAA TGCCGTCTGA AAGCCTTTCA
Tyr Lys Phe ***>
___TRANSLAT____>

2350          2360
 .     .       .     .
GACGGCATCT GTCCTGATAA TTTGATATA
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Pleas See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.6, 7.32; 530/380, 388.25, 388.4, 389.3, 389.5; 536/23.7; 424/249.1, 250.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE
search terms: FrpB protein, vaccine, N. gonorrhoeae, N. meningitidis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Vaccine, Vol 12 No 6, issued 1994, Ala'Aldeen et al, "Vaccine Potential of meningococcal FrpB: studies on surface exposure and functional attributes of common epitopes", pages 535-541, see pages 535 and 538.	1-36 --- 37-44
X, P --- Y, P	Dissertation Abstract International, Vol 56 No 2, issued August 1995, Beucher, M., "Cloning, sequencing and characterization of the gene encoding FrpB, a major iron-regulated outer membrane protein of Neisseria gonorrhoeae", page 624, see entire document.	1-2, 4, 6, 8-12 --- 3, 5, 7, 13-44

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
09 JULY 1996	25.07.96
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Infection and Immunity, Vol 56 No 4, issued April 1988, Dyer et al, "A plieotropic iron-uptake mutant of Neisseria meningitidis lacks a 70-kilodalton iron-regulating protein", pages 977-983, see page 980.	6-7, 31-32 --- 1-5, 8-30, 33-44
X, P --- Y, P	Infection and Immunity, Vol 63, No 10, issued October 1995, Pettersson et al, "Molecular Characterization of FrpB, the 70-kilodalton iron-regulated outer membrane protein of Neisseria meningitidis", pages 4181-4184, see page 4182.	1, 3, 5, 7-12 --- 2, 4, 6, 13-44

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/04, 21/08; A61K 35/18, 38/00; C07K 1/00, 14/195, 16/12; C07H 21/04; A61K 39/095

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.6, 7.32; 530/380, 388.25, 388.4, 389.3, 389.5; 536/23.7; 424/249.1, 250.1

CORRECTED VERSION

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(54) Title: ISOLATED FrpB NUCLEIC ACID MOLECULE AND VACCINE

(57) Abstract

The present invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein. The invention also provides vaccine compositions capable of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis* comprising the FrpB protein encoded by the isolated nucleic acid of the invention and a pharmaceutically acceptable carrier.

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Isolated FrpB Nucleic Acid Mol cule and Vaccine

This invention was made in the course of work supported by Public Health Service Grant U01 A131496 and the Genetics Curriculum training grant 5 T32 GM07092 from the National Institutes of Health. Protein sequencing performed at the UCLA Protein Microsequencing Facility was aided by a BRS Shared Instrumentation Grant (I S10RR05554-01) from the National Institutes of Health. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

FrpB has been described as a 70 kD major iron-regulated, outer-membrane protein common to *N. gonorrhoeae* and *N. menigitidis* (16, 21). The iron uptake systems of *N. meningitidis* and *N. gonorrhoeae* are similar (3,17).

Previous studies showed that FrpB is surface exposed and immunogenic *in vivo* (1,16, 41). Polyclonal and some monoclonal anti-FrpB antibodies recognize the denatured protein on Western blots of nearly all gonococcal and meningococcal isolates tested (16 and this invention). Other monoclonal antibodies directed against meningococcal FrpB are bactericidal and strain specific (41). Nevertheless, the size of FrpB appears to be well conserved.

FrpB is useful as a vaccine because of its surface exposure (1,16,41), partial antigenic conservation (8,16), and susceptibility to attack by bactericidal antibodies (41). The cloning and sequencing of the *frpB* gene of this invention has made possible the

production of a vaccine against infection in mammals by *N. gonorrhoeae* or *N. meningitidis*.

SUMMARY OF THE INVENTION

The present invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein.

The invention also provides a method of producing a vaccine composition that protects a mammal from infection by *N. gonorrhoeae* or *N. meningitidis* comprising combining the FrpB protein encoded by the isolated nucleic acid of the invention with a pharmaceutically acceptable carrier.

The invention further provides a vaccine composition capable of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of the invention and a pharmaceutically acceptable carrier.

In addition, the invention provides antibodies directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of the invention.

The invention also provides a method of detecting an antibody specific for *N. gonorrhoeae* or *N. meningitidis* in a sample comprising contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of the invention under conditions to form a complex between the polypeptide and the antibody; and detecting any complex so formed.

Furthermore, the invention provides a method of treating a mammal infected by *N.*

gonorrhoeae or *N. meningitidis* comprising administering to the mammal an antibody of the invention, wherein the antibody is directed to an epitope of an *N. gonorrhoeae* or *N. meningitidis* FrpB protein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 Oligonucleotide MB.3 is shown 3' to 5' and corresponds to non-coding strand. The *frpB* sequence presented in this figure is deposited with GenBank under the accession number U13980.

FIG. 2 Restriction map of *frpB* clones. The position of the *frpB* ORF is indicated below the physical map by the stippled box. Only relevant cloning sites are shown C, *Cla* I; D, *Dra* I; E, *EcoR* I; M, *Mlu* I. Also shown is the position of oligonucleotide MB.3, which was deduced from the amino-terminal amino acid sequence of the mature protein.

FIG. 3 Nucleotide sequence of the gonococcal *frpB* gene from strain FA19. Single letter codes for deduced amino acid sequence are shown below the nucleotide sequence. Asterisk indicates termination codon. Solid bar below nucleotide sequence indicates putative Fur box. Putative -10 and -35 sequences are boxed. RBS indicates ribosome binding site. Solid triangle shows *Bgl* I site of Ω insertion. Vertical arrow indicates signal peptidase I cleavage site. Inverted horizontal arrows indicate inverted repeat.

FIG. 4 Southern-blot analysis of FA19 and FA6807 DNA. Panel A was probed with pUNCH319-specific fragment. Panel B was probed with the Ω fragment. Lanes 1 contain FA19 DNA digested with HincII and lanes 2 contain FA6807 DNA digested with HincII. Ω fragment is 2kb. Molecular weight markers are shown in kilobases (kB).

FIG. 5 Western blot of FA19 and FA6807 membranes. Blot was probed with anti-FrpB monoclonal antibody, W.6. Lanes 1 and 2 are FA19; lanes 3 and 4 are FA6807. Lanes 1 and 3 contain total membranes prepared from iron-sufficient cultures; lanes 2 and 4 contain total membranes from iron-deficient cultures. Approximate locations of molecular mass standards are indicated at left in kilodaltons.

FIG 6 Growth of FA19 and FA6807 in CDM in the presence of variable concentrations of aerobactin. Graph A represents FA19; graph B represents FA6807. (filled-in Δ), 100uM citrate; (■), 2.5uM Tf; (Δ), 3uM aerobactin; (●), 1uM aerobactin; (□), 0.3uM aerobactin; and (O), no iron source.

FIG. 7 ^{55}Fe uptake from ^{55}Fe-heme and ^{55}Fe-Tf. Solid columns represent mean uptake from heme and open columns represent mean uptake from Tf. 100% uptake determined from average FA19 uptake experiment. Standard deviations are indicated by error bars. Genotypes are FA19 wild type, FA6807 (frpB), and FA6747 (tpbA).

FIG 8 Reconstruction of *frpB* in pACYC184. Relevant sites are B, *Bam*H I; C, *Cla* I; D, *Dra* I; M, *Mlu* I; and X, *Xba* I. Solid arrow represents chloramphenicol acetyl transferase (Cm), stripped arrow represents tetracycline resistance gene (Tc), solid bar represents pACYC184 origin of replication (Ori), stippled boxes represent *frpB* coding sequences, stippled arrow indicates entire *frpB* coding regions, open boxes represent DNA 5' and 3' of *frpB*. *frpB'* and *frpB"* represent partial *frpB* coding sequences.

FIG. 9 Growth of RK1065 (pACYC184) and RK1065 (pUNCH331) on heme plates. Plate 1 contains heme only. Plate 2 contains heme and d-aminolevulinic acid. A is RK1065 (pACYC184) and B is RK1065 (pUNCH331). Antibiotic discs are E., erythromyocin; N, novobiocin; and R, rifampicin.

FIG. 10 Nucleotide sequence of the gonococcal *frpB* gene from strain FA1090. The three letter codes for deduced amino acid sequence are shown below the nucleotide sequence. Three asterisks indicate termination codon.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides an isolated nucleic acid molecule that encodes an amino acid sequence comprising at least a portion of a FrpB protein. In one embodiment of this invention, the isolated nucleic acid molecule is DNA. In other embodiments of this invention, the isolated nucleic acid molecule is cDNA or RNA. In a preferred embodiment of this invention, the isolated nucleic acid molecule comprises a sequence that is the same as or substantially the same as at least a portion of the nucleotide sequence shown in Figure 3. In a more preferred embodiment, the isolated nucleic acid molecule comprises a sequence that is the same as the nucleotide sequence shown in Figure 3.

The invention also provides a FrpB protein comprising the amino acid sequence encoded by the isolated nucleic acid molecules described above. Preferably, the amino acid sequence encodes an antigenic, and more preferably, an immunogenic FrpB. As used herein, antigenic means that the FrpB induces specific antibodies in a mammal, and immunogenic means that the FrpB induces an immune response in a mammal.

As used herein, the term "FrpB" means Fe-regulated protein B and encompasses any polypeptide having an amino acid sequence identical, or substantially identical, to the amino acid sequence of a naturally-occurring FrpB, as well as antigenic fragments thereof. The FrpB nucleic acid and amino acid sequences in the various strains of *N. gonorrhoeae* and *N. meningitidis* are homologous, but exhibit slight differences in their sequences, for example, the nucleic acid and amino acid differences between the homologous strains FA19 and FA1090 shown in Figure 3 and Figure 10, respectively.

In addition, FrpB encompasses equivalent antigenic polypeptides whose amino acid sequence varies from a naturally-occurring FrpB by one or more amino acid, either

internally such as a point mutation, or by addition or deletion at the COOH terminus or NH_2 terminus or both. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by one or more substitutions, additions and/or deletions, is considered to be an equivalent sequence. Preferably, less than 25%, more preferably less than 10%, and most preferably less than 5% of the number of amino acid residues in a sequence are substituted for, added to, or deleted from the proteins of the invention.

For example, it is known to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids generally considered to be equivalent are:

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);
(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V); and
(e) Phe(F) Tyr(Y) Trp(W).

Such FrpB equivalents include analogs that induce an immune response in a mammal comparable to that of natural FrpB. In addition, such equivalents are immunologically cross-reactive with their corresponding FrpB protein.

A FrpB protein fragment preferably contains sufficient amino acid residues to define an epitope of the antigen. The fragment may, for example, be a minigene encoding only the epitope. Methods for isolating and identifying immunogenic fragments from known immunogenic proteins are described by Salfeld et al. (72) and by Isola et al. (73).

If the fragment defines a suitable epitope, but is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet

hemocyanin, Ig sequences, TrpE, and human or bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

In a preferred embodiment, FrpB of FA19 is or is an equivalent of the approximately 73 kD outer membrane FrpB protein that is part of the iron regulon of *Neisseria gonorrhoeae* or of *Neisseria meningitidis*. Determinations whether two amino acid sequences are substantially homologous may be based on FASTA searches in accordance with Pearson and Lipman (74).

The FrpB of the present invention may be prepared by methods known in the art. Such methods include, for example, (a) isolating FrpB directly from *Neisseria gonorrhoeae* or *Neisseria meningitidis;* and (b) using the nucleic acid molecule of the invention encoding FrpB to produce recombinant FrpB.

(a) Direct Isolation of FrpB:
The FrpB may be isolated directly from *Neisseria gonorrhoeae* or *Neisseria meningitidis* by methods known in the art. First, gonococcal or meningococcal outer membranes are isolated and prepared by known methods. The methods described by West and Sparling (75) and by Schryvers and Morris (76) are suitable.

The isolated membrane FrpB proteins or fragments may be solubilized by known methods, such as the addition of detergents. Commonly used detergents include Octyl-B-Glucoside, Chaps, Zwittergent 3.14 or Triton-X. The use of detergents to enhance solubility of membrane proteins is described by Jones et al. (77), Helenius et al. (78), and Hjelmeland and Chrambach (79).

The FrpB proteins or fragments are isolated from the solubilized membrane fraction by standard methods. Some suitable methods include precipitation and liquid

chromatographic protocols such as ion exchange, hydrophobic interaction and gel filtration. See, for example, Methods Enzymol. (80) and Scopes (81).

Purified material may also be obtained by separating the protein or fragment on preparative SDS-PAGE gels, slicing out the band of interest and electroeluting the protein from the polyacrylamide matrix by methods known in the art. The detergent SDS is removed from the protein by known methods, such as by dialysis or the use of a suitable column, such as the Extracti-Gel column from Pierce.

(b) Using Nucleic Acid Molecule of the Invention to Produce FrpB:
Alternatively, recombinant methods known in the art may be used for preparing FrpB. For example, FrpB may be produced from the isolated or synthesized nucleic acid molecule of the invention that encodes at least a portion of FrpB; cloning the DNA in a suitable host; expressing the DNA in the host; and harvesting FrpB. (See Sambrook et al. (82)).

Using standard methods of nucleic acid isolation, DNA can be obtained from strains that have been deposited with the American Type Culture Collection, Rockville, Maryland. FA1090 (ATCC Accession No.) was deposited on April 8, 1996, in accordance with the Budapest Treaty. Strain FA19 (ATCC Accession No. 55073) was deposited earlier on July 12, 1996, also in accordance with the Budapest Treaty.

The DNA may also be synthesized chemically from the four nucleotides in whole or in part by methods known in the art. Such methods include those described by Caruthers in Science 230, 281-285 (1985).

If necessary a full length DNA may also be produced by preparing overlapping double-stranded oligonucleotides, filling in the gaps, and ligating the ends together. The DNA may be cloned in a suitable host cell and expressed. The DNA and protein may be recovered

from the host cell. See, generally, Sambrook et al, "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987).

The invention provides a vector which comprises the nucleic acid molecule described above which encodes an amino acid sequence comprising at least a portion of FrpB. Suitable vectors comprise, but are not limited to, a plasmid or a virus. This vector may be transfected into a suitable host cell to form a host vector system for the production of FrpB or of a polypeptide having the biological activity of at least a portion of a FrpB antigenic polypeptide.

Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic vectors include plasmids from E. coli, such as colE1, pCR1, pBR322, pMB9, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13, f1, and other filamentous single-stranded DNA phages.

Vectors for expressing proteins in bacteria, especially E.coli, are also known. Such vectors include pK233 (or any of the tac family of plasmids), T7, and lambda P_L. Examples of vectors that express fusion proteins include the PATH vectors described by Dieckmann and Tzagoloff (83). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); and glutathione S-transferase (pGST) - see Gene (84) and Peptide Research (85).

Vectors useful in yeast are available. A suitable example is the 2μ plasmid.

Suitable vectors for use in mammalian cells are also known. Such vectors include well-

known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences and vectors derived from combination of plasmids and phage DNA.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg (86); S. Subramani et al (87); R.J. Kaufmann and P.A. Sharp (88); S.I. Scahill et al (89); G. Urlaub and L.A. Chasin (90).

The expression vectors preferably contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of f1 coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Suitable expression hosts include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRCI, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

VACCINES

FrpB encoded by a nucleic acid molecule of this invention has particular utility as a vaccine that protects a mammal from infection by *N. gonorrhoeae* or *N. meningitidis,* since the FrpB unexpectedly induces an effective immune response when presented to the immune system that protects from or prevents infection by *N. gonorrhoeae* or *N. meningitidis.* To protect from infection by *N. gonorrhoeae,* the FrpB is preferably substantially the same, as defined above, as at least a portion of the FrpB of *N. gonorrhoeae.* To protect from infection by *N. meningitidis,* the FrpB is preferably substantially the same, as defined above, as at least a portion of the FrpB of *N. meningitidis.* The immune response may also produce a therapeutic effect in an already infected mammal. The mammal is preferably a human.

The invention provides a vaccine composition which comprises the FrpB protein encoded by a nucleic acid of the invention and a pharmaceutically acceptable carrier, such as saline, sterile water, phosphate buffered saline solution, liposomes and emulsions. Other buffering and dispersing agents and inert non-toxic substances suitable for delivery to a mammal may be incorporated in the vaccine composition and are well known to those skilled in the art. The compositions may be sterilized by conventional sterilization techniques.

Adjuvants, which facilitate stimulation of the host's immune response, may be used in the vaccine compositions. Such adjuvants may include, for example, muramyl peptides, lymphokines, such as interferon, interleukin-1 and interleukin-6, or bacterial adjuvants. The adjuvant may comprise suitable particles onto which the mutant or wild-type FrpB protein is adsorbed, such as aluminum oxide particles. These vaccine compositions containing adjuvants may be prepared as is known in the art.

The concentration of FrpB in the composition may vary depending on, for example, fluid volume or antigenicity, and in accordance with the particular mode of administraton chosen.

The invention further provides a method of protecting a mammal against infection by *N. gonorrhoeae* or *N. meningitidis* comprising administering to the mammal the vaccine composition of the invention. The vaccine may be administered to a mammal by methods known in the art. Such methods include, for example, oral, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, or intradermal administration.

This invention also provides a method of producing the above vaccine composition by combining FrpB with a pharmaceutically acceptable carrier, and preferably, also with an adjuvant, as defined above.

FrpB ANTIBODIES

The invention provides antibodies raised against FrpB epitopes encoded by at least a portion of the isolated nucleic acid sequence of the invention. The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein (91) and the recombinant DNA method described by Huse et al. (92).

Mammals infected with *N. gonorrhoeae or N. meningitidis* may be treated by administering an antibody of the invention. Preferably, an antibody raised against a polypeptide comprising an amino acid sequence present in *N. gonorrhoeae or N. meningitidis* is preferred.

For therapeutic purposes, the antibodies are preferably neutralizing antibodies that

significantly inhibit the growth of or kill the bacterial cells *in vitro* or *in vivo*. Growth of the bacteria is significantly inhibited *in vivo* if the inhibition or neutralization is sufficient to prevent or reduce the symptoms of the disease of a mammal infected with the disease.

Neutralizing antibodies may also be used to produce anti-idiotypic antibodies useful as vaccines for immunizing mammals infected with *N. gonorrhoeae* or *N. meningitidis*. Anti-idiotypic antibodies are prepared in accordance with methods known in the art.

DETECTING FrpB USING PROBES

The invention also provides a method of detecting FrpB in a sample using a probe specific for a FrpB polypeptide. The probe may be an antibody described above. Methods are known for detecting polypeptides with antibodies. For example, a polypeptide may be immobilized on a solid support. Immobilization of the polypeptide may occur through an immobilized first antibody specific for the polypeptide. The immobilized first antibody is incubated with a sample suspected of containing the polypeptide. If present, the polypeptide binds to the first antibody.

A second antibody, also specific for the polypeptide, binds to the immobilized polypeptide. The second antibody may be labeled by methods known in the art. Non-immobilized materials are washed away, and the presence of immobilized label

indicates the presence of the polypeptide. This and other immunoassays are described by David, et al., in U.S. Patent 4,376,110 assigned to Hybritech, Inc., La Jolla, California.

The probe may also be a nucleic acid molecule that recognizes a FrpB nucleic acid molecule of the invention. Methods for determining whether a nucleic acid molecule probe recognizes a specific nucleic acid molecule in a sample are known in the art. Generally, a labeled probe that is complementary to a nucleic acid sequence suspected of being in a sample is prepared. The presence of probe hybridized to the target nucleic acid molecule indicates the presence of the nucleic acid molecule. Suitable methods are described by Schneider et al in U.S. Patent 4,882,269, which is assigned to Princeton University, and by Segev in PCT Application WO 90/01069, which is assigned to ImClone Systems Incorporated.

The probes described above are labeled in accordance with methods known in the art. Methods for labeling antibodies have been described, for example, by Hunter and Greenwood (93) and by David et al. (94). Additional methods for labeling antibodies have been described in U.S. patents 3,940,475 and 3,645,090. Methods for labeling oligonucleotide probes have been described, for example, by Leary et al (95); Renz and Kurz (96); Richardson and Gumport (97); Smith et al. (98); and Meinkoth and Wahl (99).

The label may be radioactive. Some examples of useful radioactive labels include ^{32}P, ^{125}I, ^{131}I, and ^{3}H. Use of radioactive labels have been described in U.K. 2,034,323, U.S. 4,358,535, and U.S. 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophors, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Some useful enzymatic labels include enzymes that cause a detectable change in a substrate. Some useful enzymes and their substrates include, for example, horseradish peroxidase (pyrogallol and o-phenylenediamine), beta-galactosidase (fluorescein beta-D-galactopyranoside), and alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The use of enzymatic labels have been described in U.K. 2,019,404, EP 63,879, and by Rotman (100).

Useful chromophores include, for example, fluorescent, chemiluminescent, and bioluminescent molecules, as well as dyes. Some specific chromophores useful in the present invention include, for example, fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, and luminol.

The labels may be conjugated to the antibody or nucleotide probe by methods that are well known in the art. The labels may be directly attached through a functional group on the probe. The probe either contains or can be caused to contain such a functional group. Some examples of suitable functional groups include, for example, amino, carboxyl, sulfhydryl, maleimide, isocyanate, isothiocyanate.

The label may also be conjugated to the probe by means of a ligand attached to the probe by a method described above and a receptor for that ligand attached to the label. Any of the known ligand-receptor combinations is suitable. The biotin-avidin combination is preferred.

The polypeptide of the invention may be used to detect the presence of antibodies specific for *N. gonorrhoeae* or *N. meningitidis* in a sample. The method comprises preparing a polypeptide containing a segment having an amino acid sequence that is substantially the same as a FrpB from either *N. gonorrhoeae* to detect antibodies to *N. gonorrhoeae* or *N. meningitidis* to detect antibodies to *N. meningitidis*. The polypeptide may be prepared as described above.

The sample may, for example, be from a patient suspected of being infected with *N. gonorrhoeae or N. meningitidis*. Suitable assays are known in the art, such as the standard ELISA protocol described by R.H. Kenneth (101).

Briefly, plates are coated with antigenic polypeptide at a concentration sufficient to bind detectable amounts of the antibody. After incubating the plates with the polypeptide, the plates are blocked with a suitable blocking agent, such as, for example, 10% normal goat serum. The sample, such as patient sera, is added and titered to determine the endpoint. Positive and negative controls are added simultaneously to quantitate the amount of relevant antibody present in the unknown samples. Following incubation, the samples are probed with goat anti-human Ig conjugated to a suitable enzyme. The presence of anti-polypeptide antibodies in the sample is indicated by the presence of the enzyme.

The following Examples section is set forth to aid in an understanding of the invention. This section is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXAMPLES

Strains and growth conditions. Bacterial strains used in this experiment are described in Table 1. *Neisseria* strains were routinely cultured on GCB media (Difco Laboratories) containing Kellogg's supplements I and II (29) and grown overnight at 35^{0}C in an atmosphere of 5%CO_2. Antibiotic selection employed chloramphenicol at 1µg/ml for mTn3(Cm)(51) mutagenized strains and streptomycin at 100µg/ml for Ω (44) mutagenized strains.

For western blot analysis of total membrane proteins of iron-stressed gonococci, cells

were grown in CDM as previously described (13). Cultures were made iron replete as indicated by the addition of 100uM ferric citrate.

E.coli strains were routinely cultured on Luria-Bertani (LB) media (47). Antibiotic selection was 100μg/ml ampicillin, 100μg/ml streptomycin, 40μg/ml kanamycin, and/or 30μg/ml cholramphenicol. δ-aminolevulinic acid was used at 30μg/ml and heme at 50μg/ml. *E.coli* cultures were iron stressed by the addition of 200μM 2,2-diyridyl (Sigma Chemical Co., St. Louis, MO). Deferoxamine mesylate (desferal) was obtained from Ciba-Geigy (Basel, Switzerland).

SDS-PAGE and Western Blotting. SDS-PAGE was performed in 7.5% polyacrylamide resolving gel and 4.5% polyacrylamide stacking gel. Electrophoresis was carried out at either 40 mA for one gel, or 80 mA for two gels in the discontinuous buffer system of Laemmli (32). Transfer and development were as described previously (23,61).

Preparation of polyclonal antisera and monoclonal antibodies. Preparation of polyclonal antisera was described previously (8). Anti-FrpB monoclonal antibodies were generated by methods described previously (60).

DNA isolation, digestion, and Southern blot analysis. Chromosomal DNA was purified by CsC1-gradient centrifugation according to the methods of Stern et al. (54). Plasmids were purified by either CsC1 centrifugation or according to the instructions provided in the Magic Miniprep™ DNA Purification Kit (Promega; Madison WI). Southern blotting and DNA hybridizations were performed as previously described (13). Restriction enzymes, Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD) and were used according to the manufacturer's

specifications. λ-ZapII and pBluescript II SK+ were obtained from Stratagene (La Jolla, CA).

DNA sequencing and sequence analysis. CsCl-purified pUNCH319 and pUNCH325 were used as templates for double-stranded DNA sequencing (31) using United States Biochemical Sequenase and the dideoxy chain termination procedure of Sanger et al. (48). Both dG- and dI- labeling reactions were carried out for all primers. Both strands of pUNCH319 were sequenced using vector-specific or insert-specific primers. Exonuclease III/Exo VII nested deletions (40) were generated from the *Mlu* end of pUNCH325 and vector-specific primers were used to sequence individual deletion clones. Internal primers were used to sequence gaps between clones as well as the opposite strand. DNA sequences were analyzed with the Genetics Computer Group software package (15) (University of Wisconsin).

Mutagenesis and gonococcal transformation. pHP45Ω (44) was used to insertionally inactivate *frpB*. pUNCH321 was digested with *Bgl* I and ends were repaired with Klenow. pHP45Ω was digested with *Sma* I and the 2.0kb Ω fragment was isolated from an agarose gel according to the instructions provided in the Geneclean II® Kit (Bio101 Inc. La Jolla, CA). Transformation of plasmid DNA into FA19 was as previously described (7).

Preparation of FrpB for amino-terminal sequence analysis. N-lauroylsarcosine (Sigma) insoluble membrane fractions were prepared from iron-stressed gonococcal strain UU1008 and protein concentration was determined by a bicinchoninic acid assay (BCA) (Pierce, Rockford, IL). Two hundred micrograms of protein was loaded into a preparative well of a 7.5% SDS-polyacryamide gel, poured 24 hours previously to permit TEMED (N,N,N',N'-tetramethylethylenediamine) and APS (ammonium persulfate) to evaporate. Electrophoresis was carried out at 40 mA constant current

using the discontinuous buffer system of Laemmli (32). The gel was soaked for 15 minutes in transfer buffer (13) before transferring. PVDF (polyvinylidene difluoride) membrane was placed in 100% methanol for two seconds, transferred to distilled deionized water (ddH_2O) for five minutes, and soaked in transfer buffer for 10 minutes prior to transfer. Transfer was for three and a half hours at 90mA in a submerged trans-blot apparatus (BioRad, Richmond, CA). Subsequent to transfer, the PVDF membrane was stained for five minutes in 0.1% Coomassie Brilliant Blue, 20% methanol, and 10% acetic acid to visualize proteins and destained for 10 minutes in ddH2O with one change. Filter was frozen at -20°C overnight. FrpB was identified by molecular weight and the amino-terminal amino acid sequence of the protein on the filter was determined by the Protein Microsequencing Facility at UCLA.

^{55}Fe uptake assays. Data were compiled from three individual experiments performed in triplicate on separate days. Gonococci were iron stressed as previously reported (2) prior to experimentation. SDS-PAGE and Western blotting of whole-cell lysates were routinely performed to determine that cultures were consistently and equivalently iron stressed, as evidenced by reactivity with anti-FrpB monoclonal antibody and/or anti-Tbp1 antisera. Iron-uptake assays were performed as previously reported (9) with the following modifications. Filters were blocked just prior to experimentation with 30µl, 10mg/ml BSA in 1XCDM. Assays were performed in 200µl volumes in 96 well filtration plates (MAHV Millipore, Bedford, MA) at 35°C in a 5% CO_2 atmosphere. Potassium cyanide was dissolved in 1XCDM. The vacuum manifold was from Millipore Multiscreen Assay System. Heme was used at 0.5µM, transferrin at 6.25µM, and citrate at 100µM. Membranes were air dried overnight, and the Millipore punch kit was used to separate and collect individual filters prior to counting. Data were expressed as counts per minute per µg of protein.

Preparation of aerobactin and enterobactin. Purified aerobactin and enterobactin

were the generous gift of P.E. Klebba. Aerobactin was ferrated as follows. Ferric sulfate was dissolved to 4mM in 50ml ddH_2O containing 1.5µl HCl. 400µ 4mM aerobactin was added to 400µl 4mM ferric sulfate and 80µl 0.5M Na_2HPO_4. The ferri-aerobactin was run over a CM-cellulose (Sigma, St. Louis, MO) column equilibrated in 0.05M Na_2HPO_4. The final concentration of aerobactin was determined by reading the absorbance at 400nM (24).

Iron sources. Human transferrin, human lactoferrin, bovine heme, human hemoglobin, and human haptoglobin were obtained from Sigma Chemical Co. (St. Louis, MO). ^{55}Fe hemin was purchased from the custom synthesizing facility at NEN Products Dupont (Wilmington, DE) lot number FE55.1193RS. Transferrin, lactoferrin, and citrate were ferrated with $^{55}FeC1$ as previously described (36).

RNase assay. The RNase assay was performed as previously described (71), except 0.1N HCl was used instead of 0.5N HCl.

Hemin affinity purification. Hemin agarose was purchased from Sigma Chemical Co. (St. Louis, MO). The method of affinity purification was described by Lee (33).

Bactericidal assays. Bactericidal assays were performed as described previously (18).

Cloning the gonococcal frpB gene. Sarcosyl insoluble membrane fractions from gonococcal strain UU1008 were used to obtain FrpB N-terminal amino acid sequence (see above). A degenerate oligonucleotide containing inosine (designated MB.3, shown in Fig. 1) was deduced from this sequence and used to probe a Southern blot of FA19 chromosomal DNA. Each restriction digest contained a single hybridizing band. A 5.8kb *Dra* I fragment was chosen for further analysis.

A λ-ZapII library containing *Eco*RI-linkered FA19 chromosomal *Dra* I fragments (2) was screened with oligo MB.3. Approximately one positive plaque was identified for every 10,000 plaques screened. Attempts to excise the phagemid containing the intact insert consistently resulted in deletion products smaller than pBluescript II SK$^+$ alone. Since such a large chromosomal fragment potentially contained both the *frpB* promoter and entire f*rpB* coding sequence and that the expression of FrpB might be toxic in *E.coli*, smaller fragments were subcloned into pBluescript II SK$^+$.

DNA prepared from one of the positively hybridized plaques, λfrpB-4(Fig. 2), was digested with *Eco*RI to release the insert DNA. The expected 5.8kb fragment was isolated from an agarose gel and further digested with *Cla* I to generate a 540bp, MB.3-hybridizing fragment and an approximately 5.3kb fragment which did not hybridize to MB.3. The smaller fragment ligated into pBluescript II SK$^+$ was stable in *E.coli* DH5αMCR and was designated pUNCH319. The larger fragment ligated into pBluescript II SK$^+$ generated pUNCH320. pUNCH320 caused *E.coli* DH5αMCR to grow poorly and appeared to be severely restricted in copy number. These data suggested that other sequences located 3' of *frpB* may also be toxic to *E.coli* and that further subcloning was necessary to obtain stable clones. Digestion of pUNCH320 with *Mlu* I and *Eco*R I released fragments of approximately 1.0 kb and 1.5kb, leaving a 2.8kb *Cla* I-*Mlu* I fragment attached to pBluescript II SK$^+$. This 5.8kb fragment (vector plus 2.8kb *Cla* I-*Mlu* I insert) was subsequently isolated, treated with Klenow, and re-ligated to itself to generate pUNCH325. DH5αMCR (pUNCH325) transformants were stable and the plasmid copy number apparently normal.

Nucleotide sequence and analysis of *frpB*. PCR amplification of chromosomal DNA followed by sequence analysis of clones confirmed the *Cla* I junction between pUNCH319 and pUNCH325. The combined nucleotide sequence and deduced amino

acid sequence from pUNCH319 and pUNCH325 are shown in Fig. 3. Putative promoter sequences were located upstream of a well conserved Fur box (4). A string of nine cytosine residues was noted between the putative -10 and -35 RNA-polymerase binding sites. A Shine-Dalgarno sequence starting at nucleotide 307 and ending at nucleotide 310 (Fig. 3), was located six bases before an ATG codon, the start of a 1,925bp open reading frame (ORF). This ORF encoded a protein of 713 amino acids. The predicted protein contained a typical signal sequence and characteristic Ala-X-Ala, signal peptidase I cleavage site. The first ten amino acids adjacent to the cleavage site were identical to the peptide sequence obtained from the mature FrpB protein. A classical TonB box was noted at residues 32-36. The mature protein had a calculated molecular weight of 76.6 kD and an isoeletric point of 10.38. The sequence downstream of the ORF revealed an inverted repeat but no string of T residues characteristic of rho-independent transcription termination (69). The protein terminated with an aromatic residue preceded by nine alternating hydrophobic and hydrophilic amino acids. This structure is typical of many bacterial outer membrane proteins sequenced to date (58).

GenBank homologies. Comparison of FrpB with other sequences in GenBank revealed some interesting homologies. Several regions of the predicted FrpB protein shared similarity with regions identified in other proteins as potentially important for membrane localization and/or TonB interaction. Localized homology was found between FrpB and the family of TonB-dependent outer membrane receptor proteins including BtuB (25) and FepA (35) of *E.coli* and between Tbp1 (13) and IroA (42) of *Neisseria* species. This similarity was limited to the highly conserved domains (13), and suggested that FrpB may also be a TonB-dependent receptor. More similarity was found with HemR, the hemin receptor of *Yersinia enterocolitica* (55). HemR is an iron-regulated, outer membrane protein that is also a member of the family of TonB-dependent receptor proteins. Overall the two proteins were 26% identical and 48% similar. The most notable similarity was seen with CopB, a major outer membrane

protein of *Moraxella catarrhalis* (26). Overall FrpB and CopB were 52% identical and 71% similar.

Transposon mutagenesis of *frpB*. In order to construct FrpB mutants, the gonococcal insert in pUNCH319 was ligated into pUP1(19), creating pUNCH321. The Ω fragment from pHP45Ω was ligated into a unique *Bgl* I site in pUNCH321 (Insertion site shown in Fig. 3). This DNA was reintroduced into the chromosome of gonococcal strain FA19 by transformation and allelic replacement, creating FA6807. Southern blot analysis of chromosomal DNA from FA19 and FA6807 indicated that a 450bp, MB.3-hybridizing, *Hinc*II fragment present in the parent was missing in FA6807 and a new reactive band of approximately 2.5kb was present (Fig. 4, panel A). An identical blot (Fig 4, panel B) probed with Ω, only hybridized to the 2.5kb fragment in FA6807. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with anti-FrpB monoclonal antibody W.6, confirmed that FrpB was absent from this strain (Fig. 5).

The Ω insertion in *frpB* was also introduced into FA6747 (*tbpA*::mTn3(Cm)) by transformation and allelic replacement creating FA6808. The FrpB$^-$/Tbp1$^-$ phenotype of FA6808 was confirmed by SDS-PAGE and Western blot analysis. This strain was used for FrpB function analysis as described below.

Utilization of iron sources. In an attempt to determine the function that FrpB plays in iron utilization, FA19 and FA6807 were grown in chemically-defined media (CDM) lacking iron. Aliquots of iron-stressed cultures were plated onto CDM agarose containing 10μM Desferal and GC base agar containing 50μM Desferal. Sterile 3mm discs containing either citrate, transferrin, lactoferrin, heme, hemoglobin, or hemoglobin bound to haptoglobin were positioned around each plate. One disc without any added iron source was added as a negative control. After overnight incubation, growth of both

strains was evident around all discs except the negative control.

N. gonorrhoeae can utilize aerobactin (67) and enterobactin (45) as iron sources. To determine if FrpB functioned as either an aerobactin or enterobactin receptor, FA19, FA6808, FA6747, KDF541, KDF541/pABN6, and BN1071 (Table 1) were iron stressed in CDM as above and plated onto CDM agarose containing 2.5μM 30% iron-saturated transferrin. FA6747 and FA6808 could not use Tf as an iron source because they lacked Tbp1, therefore these strains could grow only in the presence of a functional high-affinity siderophore receptor. Three sterile discs were positioned around each plate. Either 30% saturated lactoferrin (positive control for gonococcal viability) or filter-sterilized, iron-free supernatant from LG1315 pColV (aerobactin producer) or AN102 (enterobactin hyper-producer) were added to each disk. After overnight incubation, *E.coli* controls grew as expected suggesting that both siderophores were efficient at stripping iron from transferrin, the sole iron source provided in the media. FA19 grew over the entire transferrin plate as expected, however, growth of FA6808 and FA6747 was only evident around the lactoferrin disks, suggesting that the cells were viable but unable to use aerobactin or enterobactin under these conditions.

Aerobactin utilization by FA19 and FA6807 was further evaluated in chemically-defined, liquid media, employing various concentrations of purified ferri-aerobactin (Fig. 6). The aerobactin receptor-negative *E.coli* strain KDF541 and aerobactin receptor-positive *E.coli* strain KDF541(pABN6) were used as controls. These data suggested that *N. gonorrhoeae* FA19 and FA6807 used ferri-aerobactin similarly and in a concentration-dependent fashion analogous to the aerobactin receptor-negative *E.coli* control. Growth stimulation of gonococci by ferri-aerobactin required relatively high concentrations (3μM) and never attained a density equivalent to that of the Tf or citrate controls. These experiments confirmed the ability of gonococci to utilize ferri-aerobactin as an iron source *in vitro* but showed that this ability was not dependent upon a high-affinity receptor-mediated event.

^{55}Fe uptake from hemin, Tf, and citrate. Because of the high degree of similarity between HemR, a known hemin receptor in *Y.enterocolitica* and FrpB, it was analyzed whether a quantitative difference in ^{55}Fe uptake from hemin could be detected between FA19 and FA6807. Uptake of ^{55}Fe from transferrin by FA19, FA6807, and the Tbp1 mutant FA6747 were used as controls. The results indicated that while ^{55}Fe uptake from transferrin was approximately wild type in FA6807(P=.826), ^{55}Fe uptake from hemin was reduced by approximately 60% (P<0.001)(Fig. 7). Surprisingly, ^{55}Fe uptake from hemin was also significantly reduced in FA6747 (P<0.001). To determine whether the inability to use ^{55}Fe from hemin was specific to FA6807(FrpB$^-$) and FA6747 (Tbp1$^-$), ^{55}Fe uptake from hemin was assayed in other well-characterized, gonococcal mutants specifically altered in the expression of other iron-repressible proteins. The Tbp2$^-$ and Lbp$^-$ strains, FA6819 and FA6775 respectively, were also reduced in ^{55}Fe internalization from hemin (P<0.001). These data suggested that either more than one protein was involved in the internalization of hemin iron or the notable decrease in hemin-iron uptake in these mutants resulted from unanticipated, non-specific effects of each of these mutations on a separate membrane-bound, heme-iron-uptake system.

Reconstruction of *frpB* in pACYC184 and functional complementation of RK1065(*hemA*). In an attempt to determine if FrpB could function as a heme receptor, an *E.coli hemA* mutant was complemented with FrpB. Although expression of FrpB from the high copy-number vector pBluescript II SK$^+$ was toxic to *E.coli*, expression from the low copy-number vector pACYC184 was tolerated. The *frpB* reconstruction strategy is outlined in Fig. 8. Briefly, the insert from pUNCH319 was ligated into the *Cla* I and *Bam*H I sites of pACYC184, generating pUNCH330. pUNCH330 was digested with *Cla* I and the gel-purified *Cla* I-*Xba* I fragment from pUNCH325 was ligated into this site as follows. After ligating for four hours, Klenow was added to the ligation mixture for 30 minutes at room temperature to repair non-ligated *Cla* I and *Xba*

I ends. The reaction was further ligated overnight. The *frpB* clone in pACYC184 was designated pUNCH331. FrpB expression from pUNCH331 was iron repressible, suggesting regulation by *E.coli* Fur.

RK1065 is an *E.coli hemA* mutant which is unable to synthesize or internalize heme (27). Growth stimulation requires either δ-aminolevulinic acid, or heme and a functional heme receptor. Transformation of pUNCH331 into RK1065 supported growth on heme plates, whereas pACYC184 alone did not (Fig. 9). An Rnase leakage assay was performed to determine if FrpB expression altered the *E.coli* outer membrane, thereby allowing heme to simply diffuse into the cell (71). The *E.coli* strains C386 and HB101 containing pEBH21 were used as positive and negative controls respectively. No difference in leakiness was detected between RK1065 (pACYC184) and RK1065 (pUNCH331), suggesting that growth of RK1065 (pUNCH331) on heme plates was not due to a membrane perturbation gross enough to permit leakage of the periplasmic protein RNase H. Nevertheless, RK1065 (pUNCH331) was more sensitive to several hydrophobic antibiotics than the same strain with pACYC194 alone (Fig. 9). This experiment suggested that the presence of FrpB in *E.coli* probably allowed heme to enter non-specifically either by creating a pore or by perturbing the integrity of the outer membrane. Uptake of ^{55}Fe from hemin in RK1065 (pUNCH331) was not inhibited by KCN, consistent with a non-specific, non-receptor mediated mechanism of uptake.

Bactericidal Assay. In *M. catarrhalis*, CopB, the protein with the greatest similarity to FrpB, appears to play a major role in serum resistance. Mutants which are missing CopB have decreased serum resistance. Mutants which are missing CopB have decreased serum resistance and survival in a mouse model (26). Standard bactericidal assays were performed with normal human serum on FA19 and FA6807 grown under iron-limiting conditions and were unable to detect any difference in survival; both strains were completely serum resistant.

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Table 1. Bacterial strains, plasmids and phage.

Strain, plasmid or phage	Description	Source/reference
FA19	Wild type	[Mickelsen, 1981 #38]
FA6807	*frpB*::Ω(FrpB$^-$)	This study
FA6808	*frpB*::Ω tbpA::mTn3(Cm) (FrpB$^-$, Tbp1$^-$)	This study
FA6747	*tbpA*::mTn3(Cm) (Tbp1$^-$)	[Cornelissen, 1992 #13]
FA6819	Δ*tbpB* (Tbp2$^-$)	[Anderson, 1994 #2]
FA6775	*lbpA*::mTn3(Cm) (Lbp$^-$)	[Biswas, 1994 #6]
UU1008	Wild type	Zell McGee
DH5αMCR	*F$^-$ mcrA mcrB mrr* φ80*dlacZ*ΔM15 Δ(*argF-lac*)*U169 recAl endAl hsdR hsdM supE44* λ$^-$*thi-1 gyrA96 relAI*	Bethesda Research Labs
BN1071	*F$^-$, pro, trp, rslL, entA* (Ent$^-$, FepA$^+$)	[Klebba, 1982 #30]
AN102	BN1071, *leu, fepA* (Ent$^+$, FepA$^-$)	[Klebba, 1982 #30]
KDF541	BN1071, *entA, fepA* (Ent$^-$, FepA$^-$)	[Rutz, 1992 #46]
KDF541 / pABN6	(Ent$^-$, FepA$^-$,IutA$^+$, luc$^-$)	[de Lorenzo, 1987]
LG1315/ pcolV	BN1071, *cir* (IutA$^+$, luc$^+$)	[Warner, 1981 #63]
RK1065	*hemA*	R. Kadner
HB101	*F$^-$, hsd20 (r_B^-,m_B^-), recA13, ara-14, proA2, lacYl, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44,* λ$^-$	Maniatis et. al. 1982
C386	ompA lpp	[Sonntag, 1978 #53]
pACYC184	ori p15a, Cm^R, Tc^R	New England Biolabs
pBluescript II SK+	ori pMB1, Ap^R	Stragene
pHP45Ω	source for the Ω fragment (Sm^R)	[Prentki, 1984 #44]
pUP1	pHSS6 containing gonococcal uptake seqeunce (Kan^R)	[Elkins, 1991 #19]

pEBH21	pBC II SK$^+$ derivative (Cm^R)	[Hardham, 1994 #22]
pUNCH319	pBluescript II SK$^+$ containing 540bp *Eco*R I-*Cla* I fragment from λfrpB.4	This Study
pUNCH320	pBluescript II SK$^+$ containing 5.3kb *Cla* I-*Eco*RI fragment from λfrpB.4	This Study
pUNCH321	pUP1 containing 540bp *EcoR* I-*Cla* fragment from pUNCH319	This Study
pUNCH324	pUNCH321 containing Ω fragment from pHP45Ω in unique *Bgl* I site	This Study
pUNCH325	pBluescript II SK$^+$ containing 2.8kb *Cla* I-*Mlu* I fragment from pUNCH320	This Study
pUNCH330	540bp *EcoR* I-*Cla* fragment from pUNCH319 in pACYC184	This Study
pUNCH331	reassembled gonococcal *frpB* gene in pACYC184	This Study
λ ZapII	excisable lambda phage vector	Statagene

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CLAIMS

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule that encodes an amino acid sequence comprising a FrpB protein.

2. An isolated nucleic acid molecule of claim 1 comprising the nucleotide sequence of Figure 3.

3. An isolated nucleic acid molecule of claim 1 comprising the nucleotide sequence of Figure 10.

4. An isolated nucleic acid molecule of claim 1, wherein the FrpB protein is the FrpB protein of *Neisseria gonorrhoeae*.

5. An isolated nucleic acid molecule of claim 1, wherein the FrpB protein is the FrpB protein of *Neisseria meningitidis*.

6. A polypeptide encoded by the isolated nucleic acid molecule of claim 2.

7. A polypeptide encoded by the isolated nucleic acid molecule of claim 3.

8. A vector which comprises the nucleic acid molecule of claim 1.

9. A vector of claim 8, wherein the nucleic acid molecule is linked to a plasmid.

10. A host vector system for the production of a polypeptide having the biological activity of a FrpB antigenic polypeptide which comprises the vector of claim 8 in a

suitable host.

11. A host vector system of claim 10, wherein the suitable host is a bacterial cell or animal cell.

12. A method of producing a polypeptide having the biological activity of a FrpB antigenic polypeptide which comprises growing the host vector system of claim 10 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

13. A method of producing a vaccine composition that protects a mammal from infection by *N. gonorrhoeae* comprising combining the FrpB protein encoded by the isolated nucleic acid of claim 1 with a pharmaceutically acceptable carrier.

14. The method of claim 13 further comprising combining the FrpB with an effective amount of an adjuvant.

15. The method of claim 13, wherein the amino acid sequence of the polypeptide comprises the FrpB protein of *N. gonorrhoeae*.

16. The method of claim 13, wherein the mammal is a human.

17. A method of producing a vaccine composition that protects a mammal from infection by *N. meningitidis* comprising combining the FrpB protein encoded by the isolated nucleic acid of claim 1 with a pharmaceutically acceptable carrier.

18. The method of claim 17 further comprising combining the FrpB with an effective amount of an adjuvant.

19. The method of claim 17, wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. meningitidis.*

20. The method of claim 17, wherein the mammal is a human.

21. A vaccine composition capable of protecting a mammal against infection by *N. gonorrhoeae*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

22. The vaccine composition of claim 21 further comprising an effective amount of an adjuvant.

23. The vaccine composition of claim 21, wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. gonorrhoeae.*

24. The vaccine composition of claim 21, wherein the mammal is a human.

25. A vaccine composition capable of protecting a mammal against infection by *N. meningitidis*, the vaccine composition comprising the FrpB protein encoded by the isolated nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

26. The vaccine composition of claim 25 further comprising an effective amount of an adjuvant.

27. The vaccine composition of claim 25, wherein the amino acid sequence of the polypeptide comprises at least a portion of the FrpB protein of *N. meningitidis.*

28. The vaccine composition of claim 25, wherein the mammal is a human.

29. A method of protecting a mammal against infection by *N. gonorrhoeae* comprising administering to the mammal a vaccine composition of claim 21.

30. A method of protecting a mammal against infection by *N. meningitidis* comprising administering to the mammal a vaccine composition of claim 25.

31. An antibody directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of claim 2.

32. An antibody directed to an epitope of the FrpB protein encoded by the isolated nucleic acid sequence of claim 3.

33. A method of detecting an antibody specific for *N. gonorrhoeae* in a sample comprising:

(a) contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of claim 4 under conditions to form a complex between the polypeptide and the antibody; and

(b) detecting any complex so formed;

thereby detecting an antibody specific for *N. gonorrhoeae.*

34. A method of claim 33, wherein the FrpB protein is labeled with a detectable marker.

35. A method of detecting an antibody specific for *N. meningitidis* in a sample comprising:

(a) contacting the sample with a FrpB protein encoded by the isolated nucleic acid sequence of claim 5 under conditions to form a complex between the polypeptide and the antibody; and

(b) detecting any complex so formed;

thereby detecting any antibody specific for *N. meningitidis*.

36. A method of claim 35, wherein the FrpB protein is labeled with a detectable marker.

37. A method of treating a mammal infected by *N. gonorrhoeae* comprising administering to the mammal an antibody of claim 31.

38. A method of treating a mammal infected by *N. gonorrhoeae* comprising administering to the mammal an antibody of claim 32.

39. The method of claim 37 or 38 wherein the mammal is a human.

40. The method of claim 37 or 38 wherein the antibody is monoclonal.

41. A method of treating a mammal infected by *N. meningitidis* comprising administering to the mammal an antibody of claim 31.

42. A method of treating a mammal infected by *N. meningitidis* comprising administering to the mammal an antibody of claim 32.

43. The method of claim 41 or 42 wherein the mammal is a human.

44. The method of claim 41 or 42 wherein the antibody is monoclonal.

Figure 1

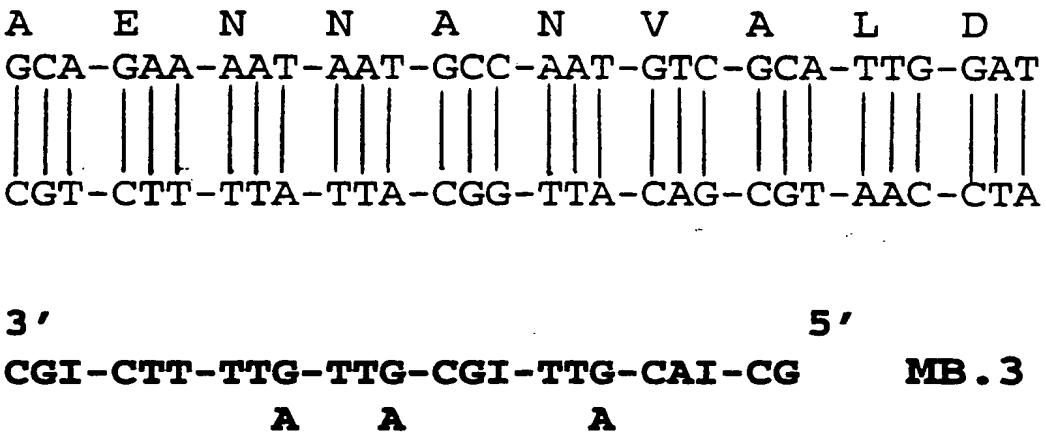
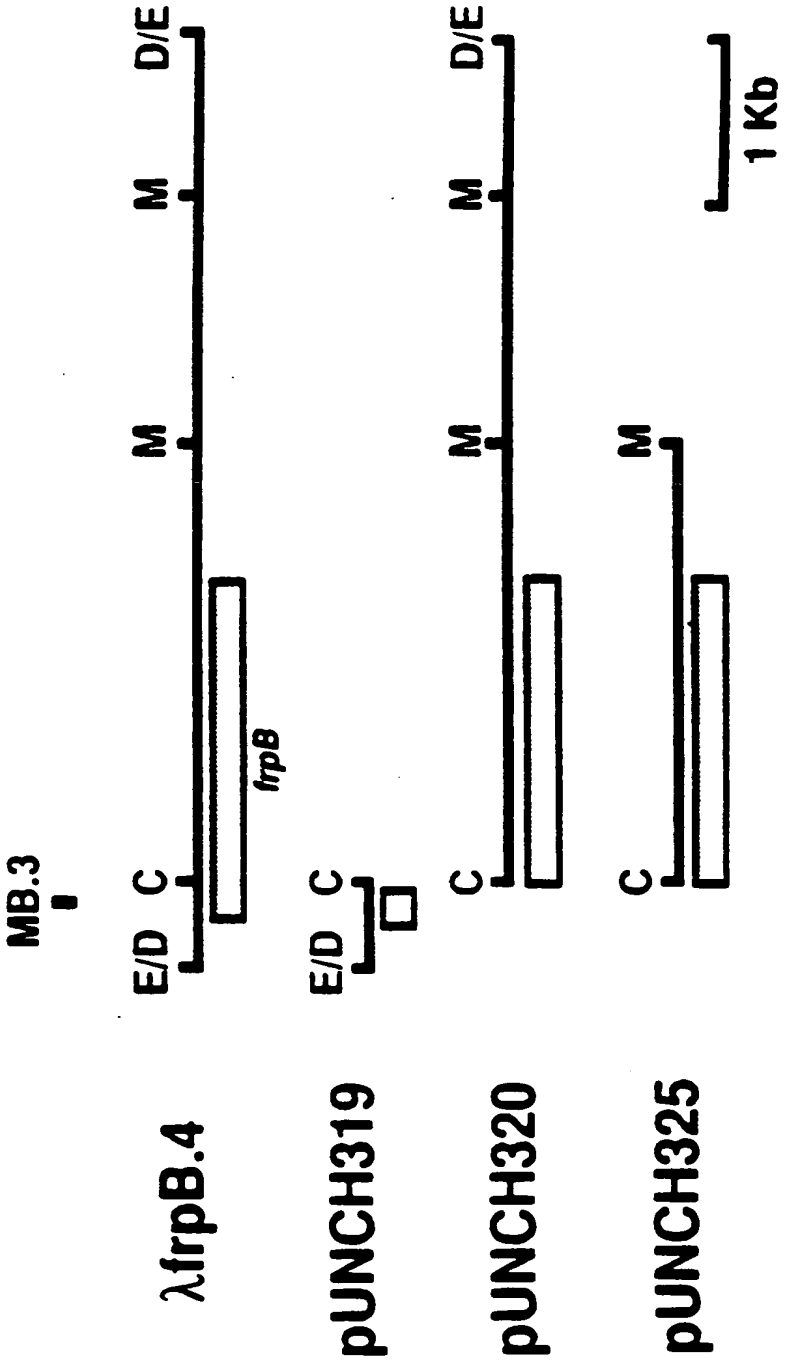
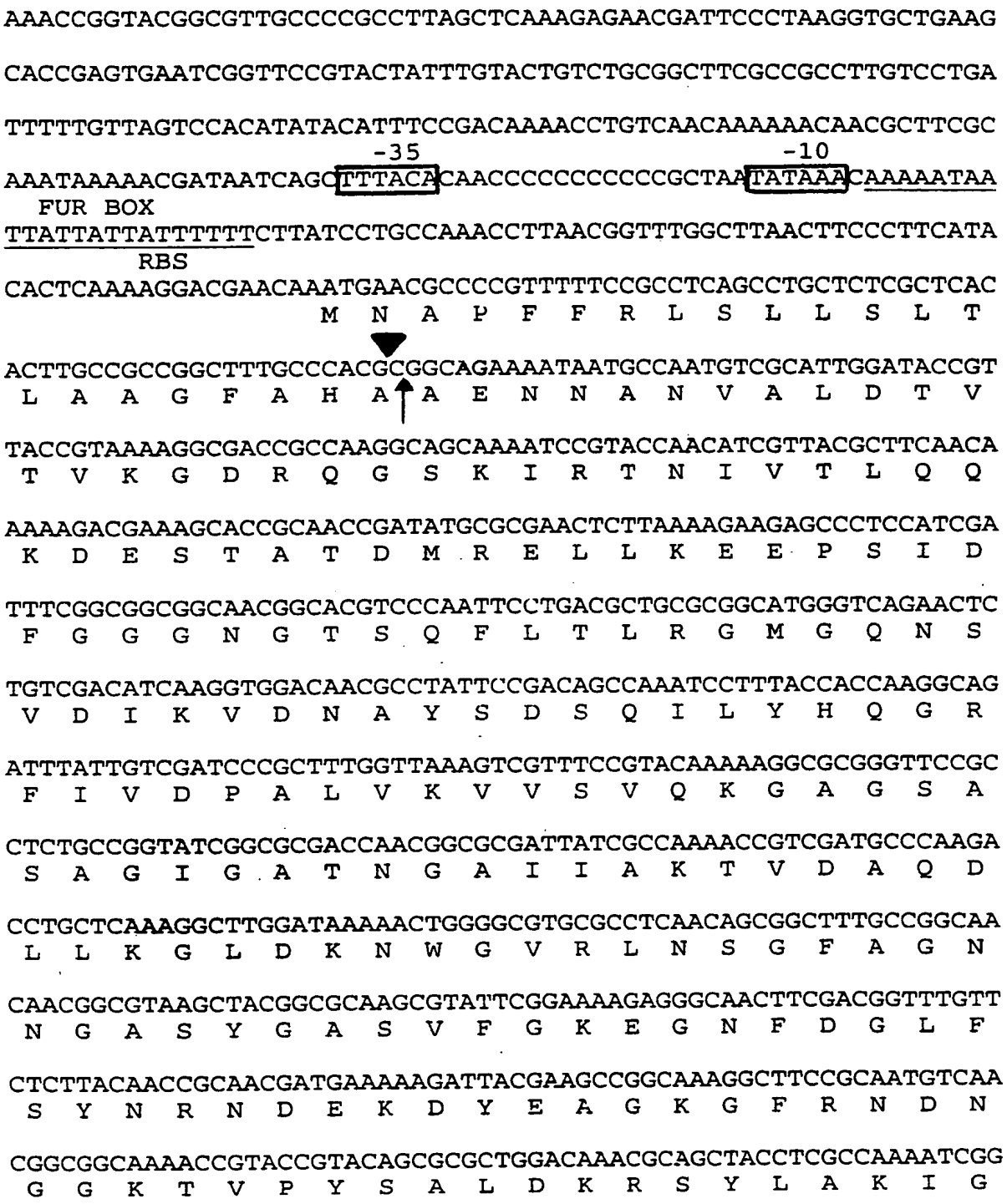


Figure 2



3/16

Figure 3-A

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TTTTTGTTAGTCCACATATACATTTCCGACAAAACCTGTCAACAAAAAACAACGCTTCGC
                    -35                          -10
AAATAAAAACGATAATCAGCTTTACACAACCCCCCCCCCCGCTAATATAAACAAAAATAA
  FUR BOX
TTATTATTATTTTTTCTTATCCTGCCAAACCTTAACGGTTTGGCTTAACTTCCCTTCATA
          RBS
CACTCAAAAGGACGAACAAATGAACGCCCCGTTTTTCCGCCTCAGCCTGCTCTCGCTCAC
                      M  N  A  P  F  F  R  L  S  L  L  S  L  T

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 V  D  I  K  V  D  N  A  Y  S  D  S  Q  I  L  Y  H  Q  G  R

ATTTATTGTCGATCCCGCTTTGGTTAAAGTCGTTTCCGTACAAAAAGGCGCGGGTTCCGC
 F  I  V  D  P  A  L  V  K  V  V  S  V  Q  K  G  A  G  S  A

CTCTGCCGGTATCGGCGCGACCAACGGCGCGATTATCGCCAAAACCGTCGATGCCCAAGA
 S  A  G  I  G  A  T  N  G  A  I  I  A  K  T  V  D  A  Q  D

CCTGCTCAAAGGCTTGGATAAAAACTGGGGCGTGCGCCTCAACAGCGGCTTTGCCGGCAA
 L  L  K  G  L  D  K  N  W  G  V  R  L  N  S  G  F  A  G  N

CAACGGCGTAAGCTACGGCGCAAGCGTATTCGGAAAAGAGGGCAACTTCGACGGTTTGTT
 N  G  A  S  Y  G  A  S  V  F  G  K  E  G  N  F  D  G  L  F

CTCTTACAACCGCAACGATGAAAAAGATTACGAAGCCGGCAAAGGCTTCCGCAATGTCAA
 S  Y  N  R  N  D  E  K  D  Y  E  A  G  K  G  F  R  N  D  N

CGGCGGCAAAACCGTACCGTACAGCGCGCTGGACAAACGCAGCTACCTCGCCAAAATCGG
 G  G  K  T  V  P  Y  S  A  L  D  K  R  S  Y  L  A  K  I  G
```

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Figure 3-B

```
AACAACCTTCGGCGACGGCGACCACCGCATCGTATTGAGCCATATGAAAGACCAACACCG
 T  T  F  G  D  G  D  H  R  I  V  L  S  H  M  K  D  Q  H  R

GGGCATCCGCACTGTGCGTGAAGAGTTTGCCGTCGGCGGCGAAAATTCACGGATAACTAT
 G  I  R  T  V  R  E  E  F  A  V  S  E  K  N  S  R  I  T  I

TAAACGCCAAGCCCCTGCCTACCGCGAAACCACACAATCCAACACCAATTTGGCGTACAC
 K  R  Q  A  P  S  Y  R  E  T  T  Q  S  N  T  N  L  A  Y  T

CGGCAAAGATTTGGGCTTTGTCGAAAAACTGGATGCCAACGCCTATGTGCTGGAAAAAAA
 G  K  D  L  G  F  V  E  K  L  D  A  N  A  Y  V  L  E  K  K

ACGCTATTCCGCCGATGACAAAGATAACGGCTACGCAGGCAATGTAAAAGGCCCCAACCA
 R  Y  S  A  D  D  K  D  N  G  Y  A  G  N  V  K  G  P  N  H

TACCCGAATCGCCACTCGGGGCATGAACTTCAACTTCGACAGCCGCCTTGCCGAACAAAC
 T  R  I  A  T  R  S  M  N  F  N  F  D  S  R  L  A  E  Q  T

CCTGTTGAAATACGGCATCAACTACCGCCATCAGGAAATCAAACCGCAAGCGTTTTTGAA
 L  L  K  Y  G  I  N  Y  R  H  Q  E  I  K  P  Q  A  F  L  N

TTCACAATTTAAAATTGAAGATAAAAAAGATGCAACTGAGGAAGATAAAAAGAAGAACCG
 S  E  F  E  I  K  D  K  E  K  A  T  N  E  E  K  K  K  N  R

TGAAAATGAAAAAATTGCCAAAGCCTACCGTCTGACCAACCCGACCAAAACCGATACCGG
 E  N  E  K  I  A  K  A  Y  R  L  T  N  P  T  K  T  D  T  G

CGCGTATATCGAAGCCATTCACGAGATTGACGGCTTTACCCTGACCGGCGGGCTGCGTTA
 A  Y  I  E  A  I  H  E  I  D  G  F  T  L  T  G  G  L  R  Y

CGACCGCTTCAAGGTGAAAACCCACGACGGCAAAACCGTTTCAAGCAGCAGCCTCAACCC
 D  R  F  K  V  K  T  H  D  G  K  T  V  S  S  S  S  L  N  P

GAGTTTCGGCGTGATTTGGCAGCCGCGCGAACACTGGAGCTTCAGCGCGAGCCACAACTA
 S  F  G  V  I  W  Q  P  R  E  H  W  S  F  S  A  S  H  N  Y

CGCCAGCCGCAGCCCGCGCCTGTATGACGCGCTGCAAACCCACGGCAAACGCGGCATCAT
 A  G  R  S  P  R  L  Y  D  A  L  Q  T  H  G  K  R  G  I  I

CTCGATTGCCGACGGCACCAAAGCCGAACGCGCGCGCAATACCGAAATCGGCTTCAACTA
 S  I  A  D  G  T  K  A  E  R  A  R  N  T  E  I  G  F  N  Y

CAACGACGGCACGTTTGCCGCAAACGGCAGCTACTTCCGGCAGACCATCAAAGACGCGCT
 N  D  G  T  F  A  A  N  G  S  Y  F  R  Q  T  I  K  D  A  L

TGCCAATCCGCAAAACCGCCACGACTCTGTCGCCGTCCGCGAAGCCGTCAACGCCGGCTA
 A  N  P  Q  N  R  H  D  S  V  A  V  R  E  A  V  N  A  G  Y
```

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Figure 3-C

```
CATCAAAAACCACGGTTACGAATTGGGCGCGTCCTACCGCACCGGCGGCCTGACCGCCAA
 I  K  N  H  G  Y  E  L  G  A  S  Y  R  T  G  G  L  T  A  K

AGTCGGCGTAAGCCGCAGCAAACCGCGCTTTTACGATACCCATCCTAAAAAACTGTTGAG
 V  G  V  S  H  S  K  P  R  F  Y  D  T  H  K  D  K  L  L  S

CGCGAACCCCGAGTTTGGCGCACAAACCGGCCGCACTTGGACGGCCTCCCTTGCCTACCG
 A  N  P  E  F  G  A  Q  V  G  R  T  W  T  A  S  L  A  Y  R

CTTCAAAAACCCGAATCTGGAAATCGGCTGGCGCGGACGCTATGTTCAAAAAGCTACGGG
 F  K  N  P  N  L  E  I  G  W  R  G  R  Y  V  Q  K  A  V  G

TTCGATATTGGCGGCAGGGCAAAAAGACCGCGACGGCAAATTGGAAAACGTTGTACGCCA
 S  I  L  A  A  G  Q  K  D  R  D  G  K  L  E  N  V  V  R  Q

AGGTTTCGGTGTGAACGATGTCTTCGCCAACTGGAAACCGCTGGGCAAAGACACGCTCAA
 G  F  G  V  N  D  V  F  A  N  W  K  P  L  G  K  D  T  L  N

TGTTAATCTTTCGGTTAACAACGTGTTCGACAAGTTCTACTATCCGCACAGCCAACGCTG
 V  N  L  S  V  N  N  V  F  D  K  F  Y  Y  P  H  S  Q  R  W

GACCAATACCCTGCCGGGCGTGGGACGTGATGTACGCCTGGGCGTGAACTACAAGTTCTA
 T  N  T  L  P  G  V  G  R  D  V  R  L  G  V  N  Y  K  F  *

AAACGCACATCCCGAAAAAATGCCGTCTGAAAGCCTTTCAGACGGCATCTGTCCTGATAA

TTTGATATA
```

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Figure 4

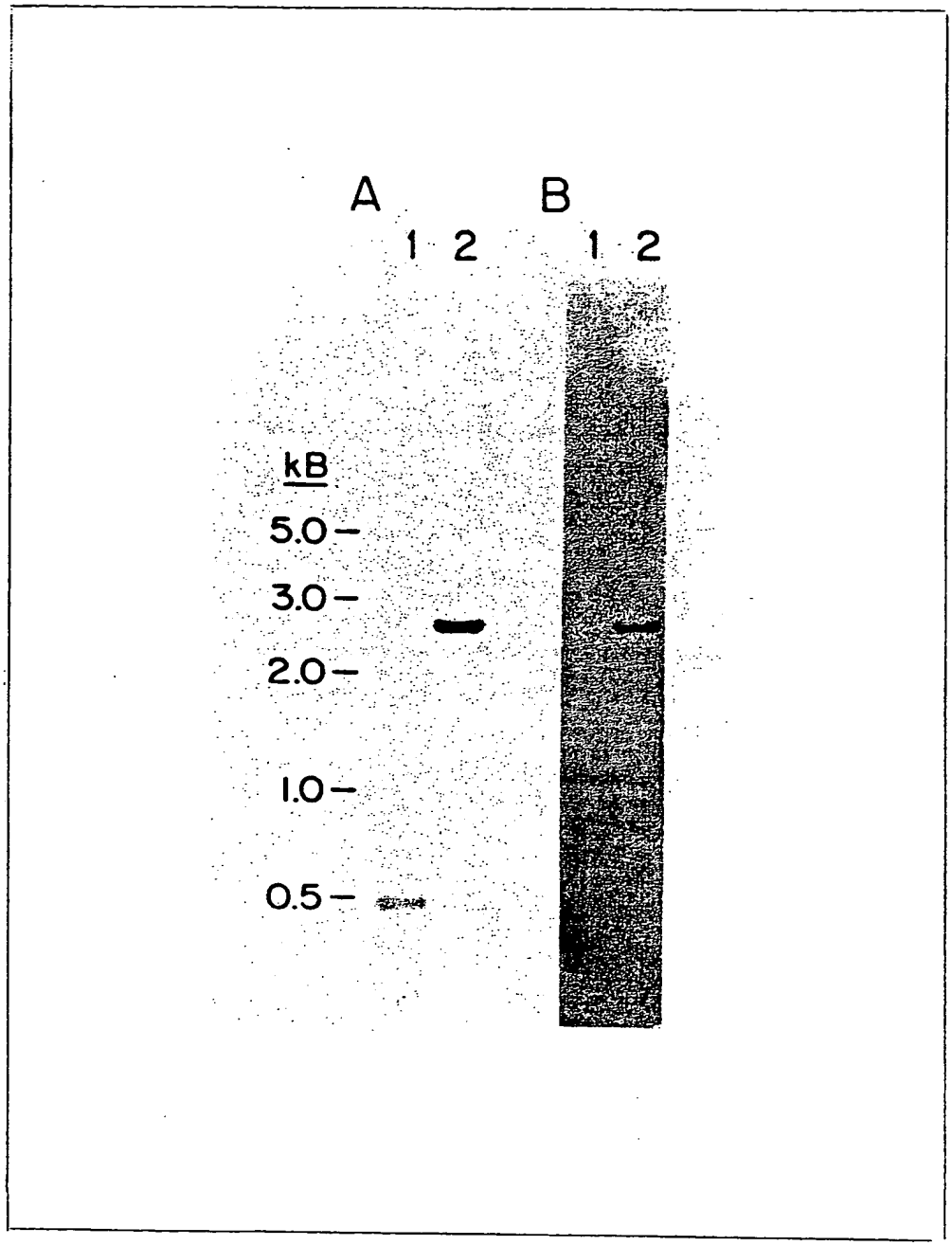

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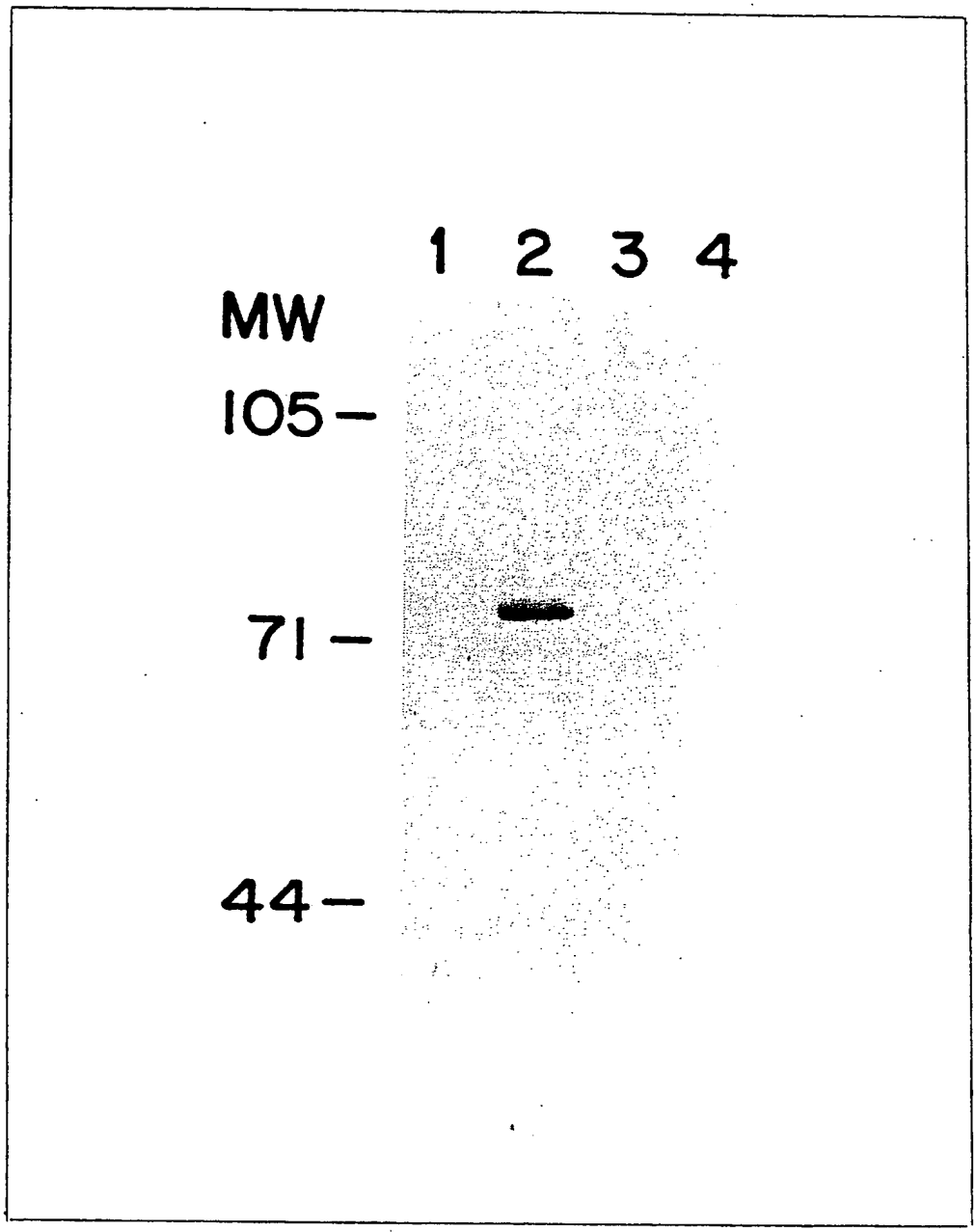
Figure 5



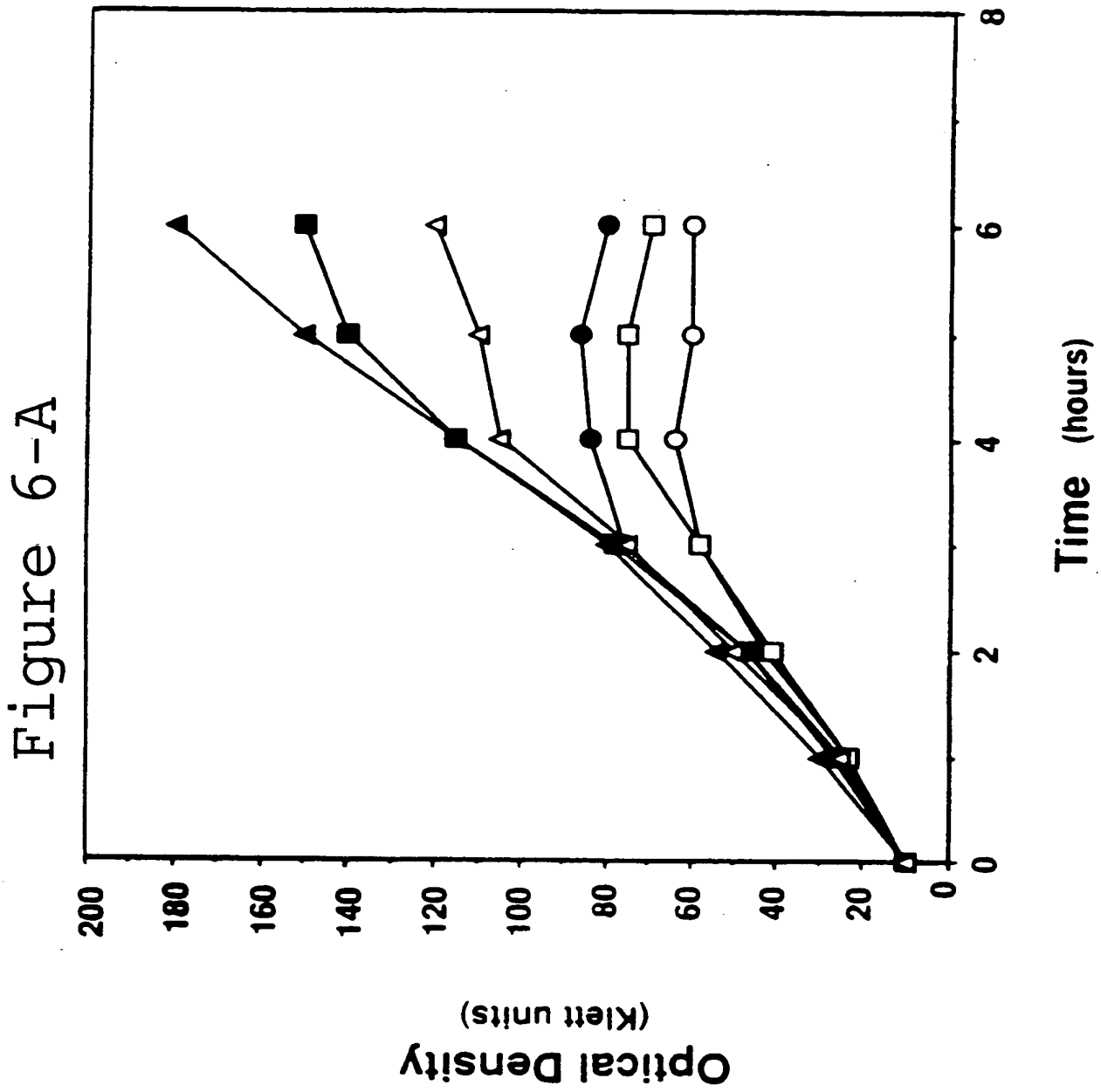


Figure 6-A

Figure 6-B

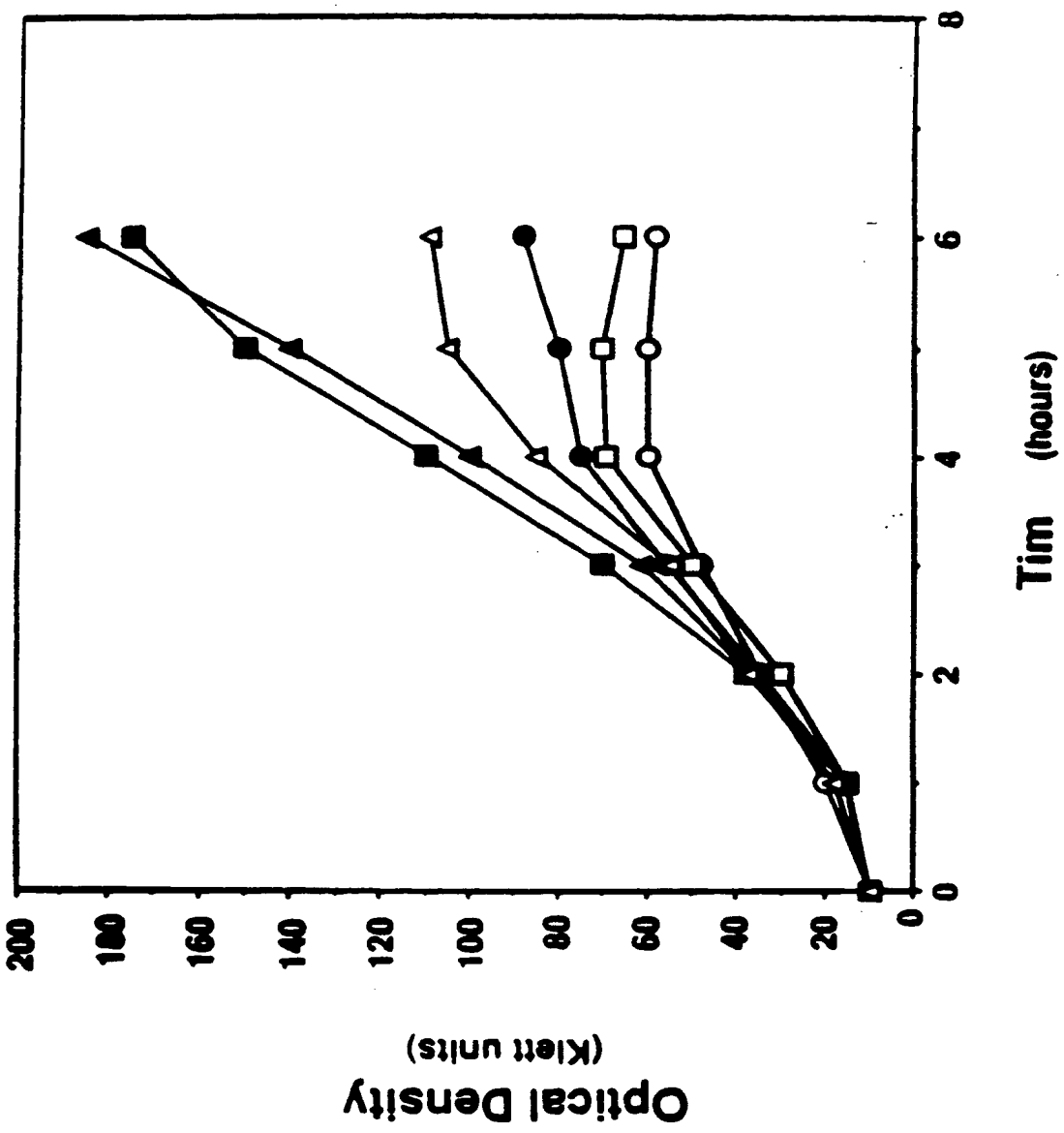


Figure 7

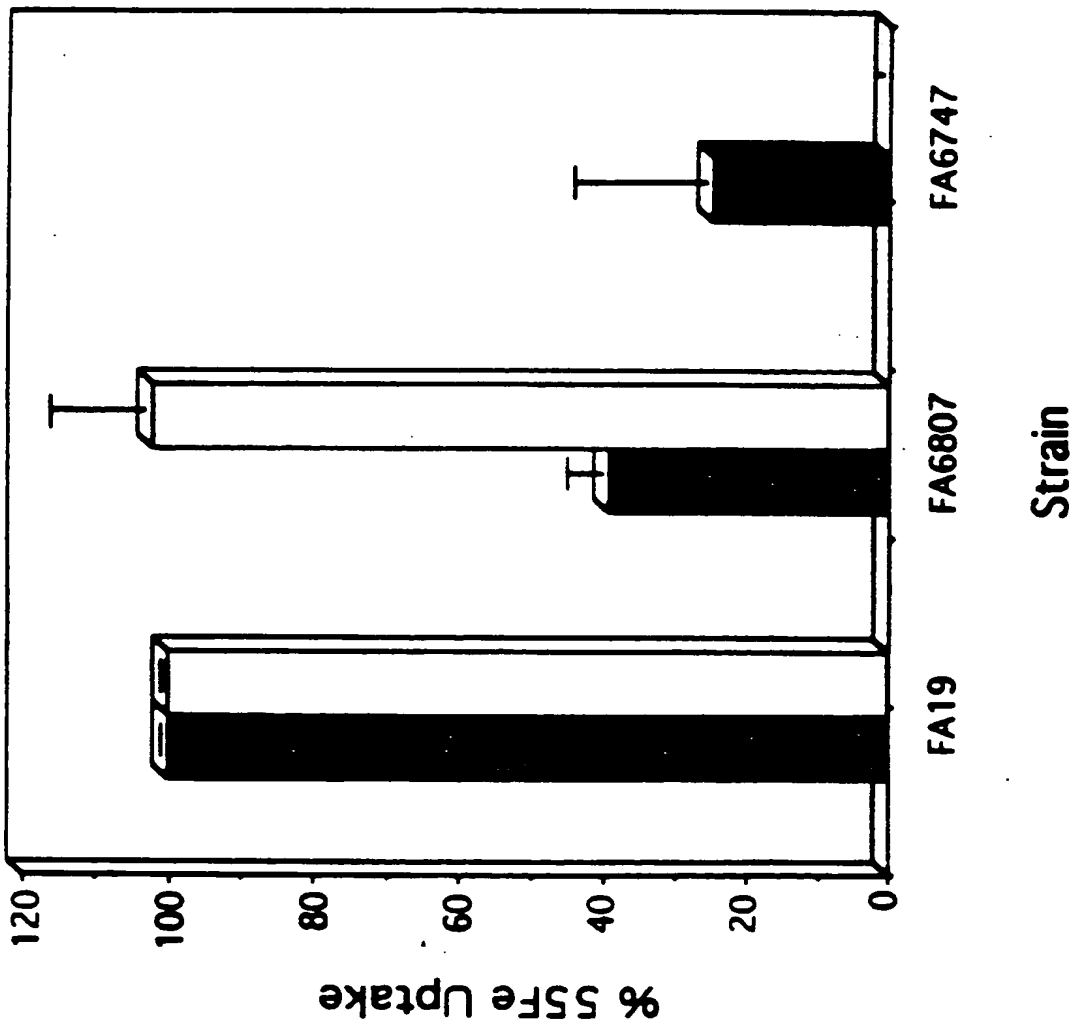


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Figure 8

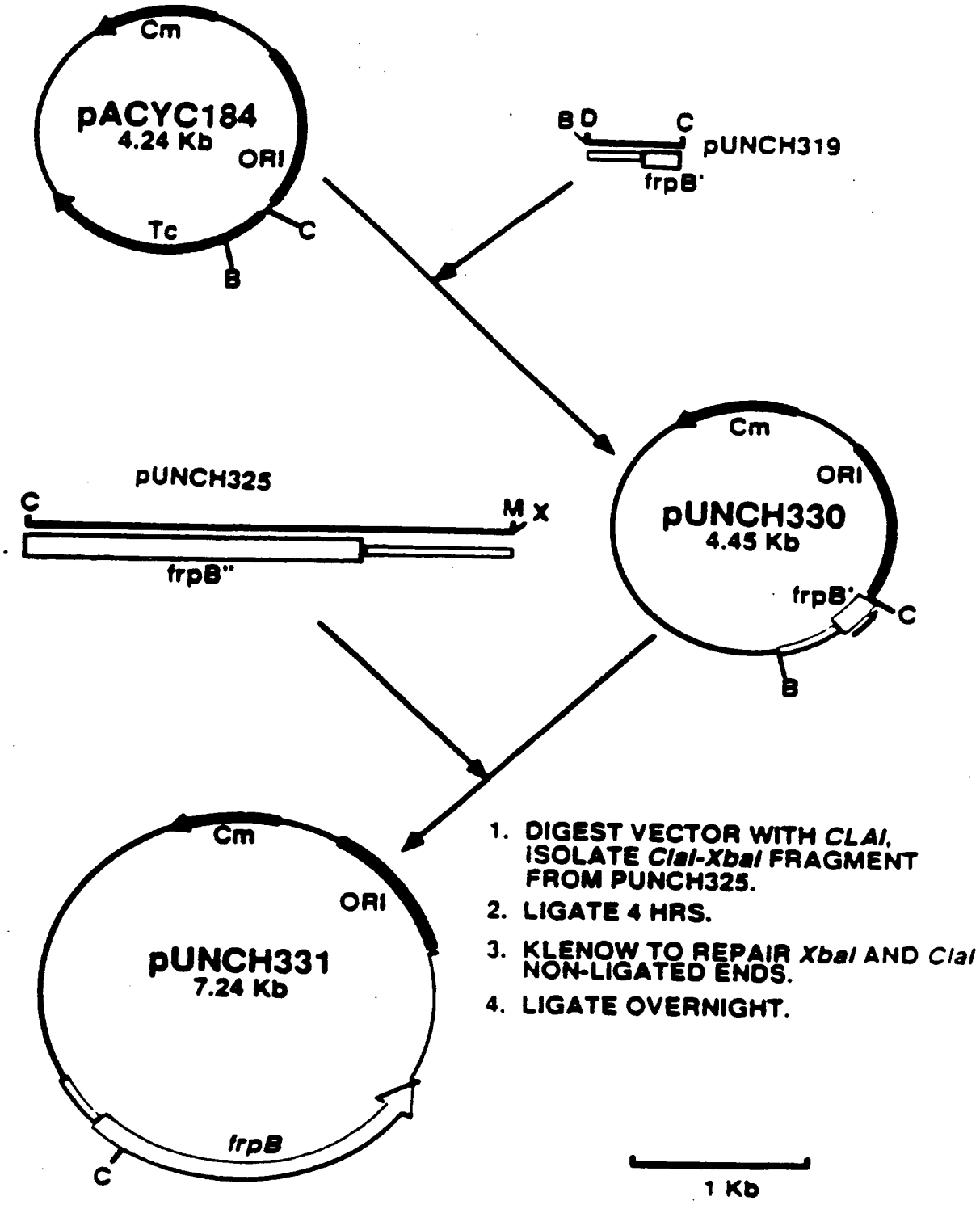

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Figure 9

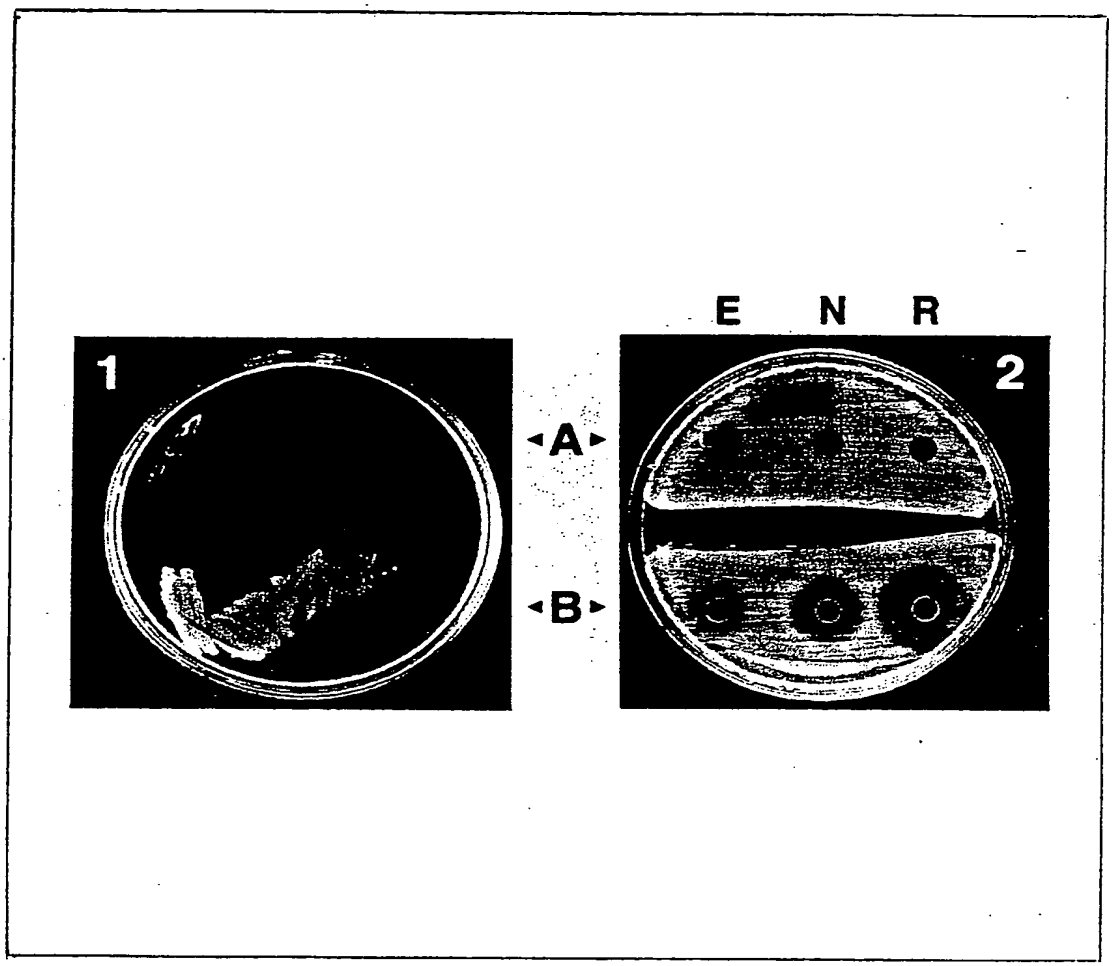

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Figure 10-A

```
                                                                AACAAAAAACAACG

CTTCGCAAATAAAAACGATAATCAGCTTTACACAACCCCCCCCCCCGCTAATATAAACAAAAA

TAATTATTATTATTTTTTCTTATCCTGCCAAACCTTAACGGTTTGGCTTAACTTCCCTTCATA

CACTCAAAAGGACGAACAA ATG AAC GCC CCG TTT TTC CGC CTC AGC CTG CTC
                    TAC TTG CGG GGC AAA AAG GCG GAG TCG GAC GAG
                    Met Asn Ala Pro Phe Phe Arg Leu Ser Leu Leu

TCG CTC ACA CTT GCC GCC GGC TTT GCC CAC GCG GCA GAA AAT AAT GCC
AGC GAG TGT GAA CGG CGG CCG AAA CGG GTG CGC CGT CTT TTA TTA CGG
Ser Leu Thr Leu Ala Ala Gly Phe Ala His Ala Ala Glu Asn Asn Ala

AAT GTC GCA TTG GAT ACC GTT ACC GTA AAA GGC GAC CGC CAA GGC AGC
TTA CAG CGT AAC CTA TGG CAA TGG CAT TTT CCG CTG GCG GTT CCG TCG
Asn Val Ala Leu Asp Thr Val Thr Val Lys Gly Asp Arg Gln Gly Ser

AAA ATC CGT ACC AAC ATC GTT ACG CTT CAA CAA AAA GAC GAA AGC ACC
TTT TAG GCA TGG TTG TAG CAA TGC GAA GTT GTT TTT CTG CTT TCG TGG
Lys Ile Arg Thr Asn Ile Val Thr Leu Gln Gln Lys Asp Glu Ser Thr

GCA ACC GAT ATG CGC GAA CTC TTA AAA GAA GAG CCC TCC ATC GAT TTC
CGT TGG CTA TAC GCG CTT GAG AAT TTT CTT CTC GGG AGG TAG CTA AAG
Ala Thr Asp Met Arg Glu Leu Leu Lys Glu Glu Pro Ser Ile Asp Phe

GGC GGC GGC AAC GGC ACG TCC CAA TTC CTG ACG CTG CGC GGC ATG GGT
CCG CCG CCG TTG CCG TGC AGG GTT AAG GAC TGC GAC GCG CCG TAC CCA
Gly Gly Gly Asn Gly Thr Ser Gln Phe Leu Thr Leu Arg Gly Met Gly

CAG AAC TCT GTC GAC ATC AAG GTG GAC AAC GCC TAT TCC GAC AGC CAA
GTC TTG AGA CAG CTG TAG TTC CAC CTG TTG CGG ATA AGG CTG TCG GTT
Gln Asn Ser Val Asp Ile Lys Val Asp Asn Ala Tyr Ser Asp Ser Gln

ATC CTT TAC CAC CAA GGC AGA TTT ATT GTC GAT CCC GCT TTG GTT AAA
TAG GAA ATG GTG GTT CCG TCT AAA TAA CAG CTA GGG CGA AAC CAA TTT
Ile Leu Tyr His Gln Gly Arg Phe Ile Val Asp Pro Ala Leu Val Lys

GTC GTT TCC GTA CAA AAA GGC GCG GGT TCC GCC TCT GCC GGT ATC GGC
CAG CAA AGG CAT GTT TTT CCG CGC CCA AGG CGG AGA CGG CCA TAG CCG
Val Val Ser Val Gln Lys Gly Ala Gly Ser Ala Ser Ala Gly Ile Gly

GCG ACC AAC GGC GCG ATT ATC GCC AAA ACC GTC GAT GCC CAA GAC CTG
CGC TGG TTG CCG CGC TAA TAG CGG TTT TGG CAG CTA CGG GTT CTG GAC
Ala Thr Asn Gly Ala Ile Ile Ala Lys Thr Val Asp Ala Gln Asp Leu

CTC AAA GGC TTG GAT AAA AAC TGG GGC GTG CGC CTC AAC AGC GGC TTT
GAG TTT CCG AAC CTA TTT TTG ACC CCG CAC GCG GAG TTG TCG CCG AAA
Leu Lys Gly Leu Asp Lys Asn Trp Gly Val Arg Leu Asn Ser Gly Phe
```

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Figure 10-B

```
GCC GGC AAC AAC GGC GTA AGC TAC GGC GCA AGC GTA TTC GGA AAA GAG
CGG CCG TTG TTG CCG CAT TCG ATG CCG CGT TCG CAT AAG CCT TTT CTC
Ala Gly Asn Asn Gly Val Ser Tyr Gly Ala Ser Val Phe Gly Lys Glu

GGC AAC TTC GAC GGT TTG TTC TCT TAC AAC CGC AAC GAT GAA AAA GAT
CCG TTG AAG CTG CCA AAC AAG AGA ATG TTG GCG TTG CTA CTT TTT CTA
Gly Asn Phe Asp Gly Leu Phe Ser Tyr Asn Arg Asn Asp Glu Lys Asp

TAC GAA GCC GGC AAA GGC TTC CGC AAT GTC AAC GGC GGC AAA ACC GTA
ATG CTT CGG CCG TTT CCG AAG GCG TTA CAG TTG CCG CCG TTT TGG CAT
Tyr Glu Ala Gly Lys Gly Phe Arg Asn Val Asn Gly Gly Lys Thr Val

CCG TAC AGC GCG CTG GAC AAA CGC AGC TAC CTC GCC AAA ATC GGA ACA
GGC ATG TCG CGC GAC CTG TTT GCG TCG ATG GAG CGG TTT TAG CCT TGT
Pro Tyr Ser Ala Leu Asp Lys Arg Ser Tyr Leu Ala Lys Ile Gly Thr

ACC TTC GGC GAC GGC GAC CAC CGC ATC GTA TTG AGC CAT ATG AAA GAC
TGG AAG CCG CTG CCG CTG GTG GCG TAG CAT AAC TCG GTA TAC TTT CTG
Thr Phe Gly Asp Gly Asp His Arg Ile Val Leu Ser His Met Lys Asp

CAA CAC CGG GGC ATC CGC ACT GTG CGT GAA GAG TTT GCC GTC GGC GGC
GTT GTG GCC CCG TAG GCG TGA CAC GCA CTT CTC AAA CGG CAG CCG CCG
Gln His Arg Gly Ile Arg Thr Val Arg Glu Glu Phe Ala Val Gly Gly

GAA AAT TCA CGG ATA ACT ATT AAA CGC CAA GCC CCT GCC TAC CGC GAA
CTT TTA AGT GCC TAT TGA TAA TTT GCG GTT CGG GGA CGG ATG GCG CTT
Glu Asn Ser Arg Ile Thr Ile Lys Arg Gln Ala Pro Ala Tyr Arg Glu

ACC ACA CAA TCC AAC ACC AAT TTG GCG TAC ACC GGC AAA GAT TTG GGC
TGG TGT GTT AGG TTG TGG TTA AAC CGC ATG TGG CCG TTT CTA AAC CCG
Thr Thr Gln Ser Asn Thr Asn Leu Ala Tyr Thr Gly Lys Asp Leu Gly

TTT GTC GAA AAA CTG GAT GCC AAC GCC TAT GTG CTG GAA AAA AAA CGC
AAA CAG CTT TTT GAC CTA CGG TTG CGG ATA CAC GAC CTT TTT TTT GCG
Phe Val Glu Lys Leu Asp Ala Asn Ala Tyr Val Leu Glu Lys Lys Arg

TAT TCC GCC GAT GAC AAA GAT AAC GGC TAC GCA GGC AAT GTA AAA GGC
ATA AGG CGG CTA CTG TTT CTA TTG CCG ATG CGT CCG TTA CAT TTT CCG
Tyr Ser Ala Asp Asp Lys Asp Asn Gly Tyr Ala Gly Asn Val Lys Gly

CCC AAC CAT ACC CGA ATC GCC ACT CGG GGC ATG AAC TTC AAC TTC GAC
GGG TTG GTA TGG GCT TAG CGG TGA GCC CCG TAC TTG AAG TTG AAG CTG
Pro Asn His Thr Arg Ile Ala Thr Arg Gly Met Asn Phe Asn Phe Asp

AGC CGC CTT GCC GAA CAA ACC CTG TTG AAA TAC GGC ATC AAC TAC CGC
TCG GCG GAA CGG CTT GTT TGG GAC AAC TTT ATG CCG TAG TTG ATG GCG
Ser Arg Leu Ala Glu Gln Thr Leu Leu Lys Tyr Gly Ile Asn Tyr Arg
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Figure 10-C

```
CAT CAG GAA ATC AAA CCG CAA GCG TTT TTG AAT TCA CAA TTT AAA ATT
GTA GTC CTT TAG TTT GGC GTT CGC AAA AAC TTA AGT GTT AAA TTT TAA
His Gln Glu Ile Lys Pro Gln Ala Phe Leu Asn Ser Gln Phe Lys Ile

GAA GAT AAA AAA GAT GCA ACT GAG GAA GAT AAA AAG AAG AAC CGT GAA
CTT CTA TTT TTT CTA CGT TGA CTC CTT CTA TTT TTC TTC TTG GCA CTT
Glu Asp Lys Lys Asp Ala Thr Glu Glu Asp Lys Lys Lys Asn Arg Glu

AAT GAA AAA ATT GCC AAA GCC TAC CGT CTG ACC AAC CCG ACC AAA ACC
TTA CTT TTT TAA CGG TTT CGG ATG GCA GAC TGG TTG GGC TGG TTT TGG
Asn Glu Lys Ile Ala Lys Ala Tyr Arg Leu Thr Asn Pro Thr Lys Thr

GAT ACC GGC GCG TAT ATC GAA GCC ATT CAC GAG ATT GAC GGC TTT ACC
CTA TGG CCG CGC ATA TAG CTT CGG TAA GTG CTC TAA CTG CCG AAA TGG
Asp Thr Gly Ala Tyr Ile Glu Ala Ile His Glu Ile Asp Gly Phe Thr

CTG ACC GGC GGG CTG CGT TAC GAC CGC TTC AAG GTG AAA ACC CAC GAC
GAC TGG CCG CCC GAC GCA ATG CTG GCG AAG TTC CAC TTT TGG GTG CTG
Leu Thr Gly Gly Leu Arg Tyr Asp Arg Phe Lys Val Lys Thr His Asp

GGC AAA ACC GTT TCA AGC AGC AGC CTC AAC CCG AGT TTC GGC GTG ATT
CCG TTT TGG CAA AGT TCG TCG TCG GAG TTG GGC TCA AAG CCG CAC TAA
Gly Lys Thr Val Ser Ser Ser Ser Leu Asn Pro Ser Phe Gly Val Ile

TGG CAG CCG CGC GAA CAC TGG AGC TTC AGC GCG AGC CAC AAC TAC GCC
ACC GTC GGC GCG CTT GTG ACC TCG AAG TCG CGC TCG GTG TTG ATG CGG
Trp Gln Pro Arg Glu His Trp Ser Phe Ser Ala Ser His Asn Tyr Ala

AGC CGC AGC CCG CGC CTG TAT GAC GCG CTG CAA ACC CAC GGC AAA CGC
TCG GCG TCG GGC GCG GAC ATA CTG CGC GAC GTT TGG GTG CCG TTT GCG
Ser Arg Ser Pro Arg Leu Tyr Asp Ala Leu Gln Thr His Gly Lys Arg

GGC ATC ATC TCG ATT GCC GAC GGC ACC AAA GCC GAA CGC GCG CGC AAT
CCG TAG TAG AGC TAA CGG CTG CCG TGG TTT CGG CTT GCG CGC GCG TTA
Gly Ile Ile Ser Ile Ala Asp Gly Thr Lys Ala Glu Arg Ala Arg Asn

ACC GAA ATC GGC TTC AAC TAC AAC GAC GGC ACG TTT GCC GCA AAC GGC
TGG CTT TAG CCG AAG TTG ATG TTG CTG CCG TGC AAA CGG CGT TTG CCG
Thr Glu Ile Gly Phe Asn Tyr Asn Asp Gly Thr Phe Ala Ala Asn Gly

AGC TAC TTC CGG CAG ACC ATC AAA GAC GCG CTT GCC AAT CCG CAA AAC
TCG ATG AAG GCC GTC TGG TAG TTT CTG CGC GAA CGG TTA GGC GTT TTG
Ser Tyr Phe Arg Gln Thr Ile Lys Asp Ala Leu Ala Asn Pro Gln Asn

CGC CAC GAC TCT GTC GCC GTC CGC GAA GCC GTC AAC GCC GGC TAC ATC
GCG GTG CTG AGA CAG CGG CAG GCG CTT CGG CAG TTG CGG CCG ATG TAG
Arg His Asp Ser Val Ala Val Arg Glu Ala Val Asn Ala Gly Tyr Ile
```

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Figure 10-D

```
AAA AAC CAC GGT TAC GAA TTG GGC GCG TCC TAC CGC ACC GGC GGC CTG
TTT TTG GTG CCA ATG CTT AAC CCG CGC AGG ATG GCG TGG CCG CCG GAC
Lys Asn His Gly Tyr Glu Leu Gly Ala Ser Tyr Arg Thr Gly Gly Leu

ACC GCC AAA GTC GGC GTA AGC CGC AGC AAA CCG CGC TTT TAC GAT ACC
TGG CGG TTT CAG CCG CAT TCG GCG TCG TTT GGC GCG AAA ATG CTA TGG
Thr Ala Lys Val Gly Val Ser Arg Ser Lys Pro Arg Phe Tyr Asp Thr

CAT CCT AAA AAA CTG TTG AGC GCG AAC CCC GAG TTT GGC GCA CAA ACC
GTA GGA TTT TTT GAC AAC TCG CGC TTG GGG CTC AAA CCG CGT GTT TGG
His Pro Lys Lys Leu Leu Ser Ala Asn Pro Glu Phe Gly Ala Gln Thr

GGC CGC ACT TGG ACG GCC TCC CTT GCC TAC CGC TTC AAA AAC CCG AAT
CCG GCG TGA ACC TGC CGG AGG GAA CGG ATG GCG AAG TTT TTG GGC TTA
Gly Arg Thr Trp Thr Ala Ser Leu Ala Tyr Arg Phe Lys Asn Pro Asn

CTG GAA ATC GGC TGG CGC GGA CGC TAT GTT CAA AAA GCT ACG GGT TCG
GAC CTT TAG CCG ACC GCG CCT GCG ATA CAA GTT TTT CGA TGC CCA AGC
Leu Glu Ile Gly Trp Arg Gly Arg Tyr Val Gln Lys Ala Thr Gly Ser

ATA TTG GCG GCA GGG CAA AAA GAC CGC GAC GGC AAA TTG GAA AAC GTT
TAT AAC CGC CGT CCC GTT TTT CTG GCG CTG CCG TTT AAC CTT TTG CAA
Ile Leu Ala Ala Gly Gln Lys Asp Arg Asp Gly Lys Leu Glu Asn Val

GTA CGC CAA GGT TTC GGT GTG AAC GAT GTC TTC GCC AAC TGG AAA CCG
CAT GCG GTT CCA AAG CCA CAC TTG CTA CAG AAG CGG TTG ACC TTT GGC
Val Arg Gln Gly Phe Gly Val Asn Asp Val Phe Ala Asn Trp Lys Pro


CTG GGC AAA GAC ACG CTC AAT GTT AAT CTT TCG GTT AAC AAC GTG TTC
GAC CCG TTT CTG TGC GAG TTA CAA TTA GAA AGC CAA TTG TTG CAC AAG
Leu Gly Lys Asp Thr Leu Asn Val Asn Leu Ser Val Asn Asn Val Phe

GAC AAG TTC TAC TAT CCG CAC AGC CAA CGC TGG ACC AAT ACC CTG CCG
CTG TTC AAG ATG ATA GGC GTG TCG GTT GCG ACC TGG TTA TGG GAC GGC
Asp Lys Phe Tyr Tyr Pro His Ser Gln Arg Trp Thr Asn Thr Leu Pro

GGC GTG GGA CGT GAT GTA CGC CTG GGC GTG AAC TAC AAG TTC TAA AAC
CCG CAC CCT GCA CTA CAT GCG GAC CCG CAC TTG ATG TTC AAG ***
Gly Val Gly Arg Asp Val Arg Leu Gly Val Asn Tyr Lys Phe

GCACATCCCG AAAAAATGCC GTCTGAAAGC CTTTCAGACG GCATCTGTCC TGATAATTTG

ATATA
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.6, 7.32; 530/380, 388.25, 388.4, 389.3, 389.5; 536/23.7; 424/249.1, 250.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE
search terms: FrpB protein, vaccine, N. gonorrhoeae, N. meningitidis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Vaccine, Vol 12 No 6, issued 1994, Ala'Aldeen et al, "Vaccine Potential of meningococcal FrpB: studies on surface exposure and functional attributes of common epitopes", pages 535-541, see pages 535 and 538.	1-36 --- 37-44
X; P --- Y, P	Dissertation Abstract International, Vol 56 No 2, issued August 1995, Beucher, M., "Cloning, sequencing and characterization of the gene encoding FrpB, a major iron-regulated outer membrane protein of Neisseria gonorrhoeae", page 624, see entire document.	1-2, 4, 6, 8-12 --- 3, 5, 7, 13-44

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
09 JULY 1996	25.07.96
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JULIE REEVES Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Infection and Immunity, Vol 56 No 4, issued April 1988, Dyer et al, "A plieotropic iron-uptake mutant of Neisseria meningitidis lacks a 70-kilodalton iron-regulating protein", pages 977-983, see page 980.	6-7, 31-32 --- 1-5, 8-30, 33-44
X, P --- Y, P	Infection and Immunity, Vol 63, No 10, issued October 1995, Pettersson et al, "Molecular Characterization of FrpB, the 70-kilodalton iron-regulated outer membrane protein of Neisseria meningitidis", pages 4181-4184, see page 4182.	1, 3, 5, 7-12 --- 2, 4, 6, 13-44

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04774

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/04, 21/08; A61K 35/18, 38/00; C07K 1/00, 14/195, 16/12; C07H 21/04; A61K 39/095

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.6, 7.32; 530/380, 388.25, 388.4, 389.3, 389.5; 536/23.7; 424/249.1, 250.1